Cysteamine restores glutathione redox status in cultured cystinotic proximal tubular epithelial cells

Martijn J. Wilmer, Leo A.J. Kluijtmans, Thea J. van der Velden, Peter H. Willems, Peter G. Scheffer, Rosalinde Masereeuw, Leo A. Monnens, Lambertus P. van den Heuvel, Elena N. Levchenco

1. Introduction

Since cysteamine therapy has become available for patients with nephropathic cystinosis (MIM219800) in the early 1980s, quality of life for these patients has greatly improved [1,2]. The most frequent and most severe form, infantile cystinosis, is characterized by lysosomal cystine accumulation and results in renal Fanconi syndrome progressing to end stage renal disease in the majority of patients. The most common treatment involves intracellular cystine depletion by cysteamine, delaying the development of end stage renal disease by a yet elusive mechanism. However, cystine depletion does not arrest the disease nor cures Fanconi syndrome in patients, indicating involvement of other yet unknown pathologic pathways. Using a newly developed proximal tubular epithelial cell model from cystinotic patients, we investigate the effect of cystine accumulation and cysteamine on both glutathione and ATP metabolism. In addition to the expected increase in cystine and defective sodium-dependent phosphate reabsorption, we observed less negative glutathione redox status and decreased intracellular ATP levels. No differences between control and cystinosis cell lines were observed with respect to protein turnover, albumin uptake, cytosolic and mitochondrial ATP production, total glutathione levels, protein oxidation and lipid peroxidation. Cysteamine treatment increased total glutathione in both control and cystinotic cells and normalized cystine levels and glutathione redox status in cystinotic cells. However, cysteamine did not improve decreased sodium-dependent phosphate uptake. Our data implicate that cysteamine increases total glutathione and restores glutathione redox status in cystinosis, which is a positive side-effect of this agent next to cystine depletion. This beneficial effect points to a potential role of cysteamine as anti-oxidant for other renal disorders associated with enhanced oxidative stress.

Mutations in the CTNS gene, encoding for lysosomal cystine transporter cystinosin are the cause of cystinosis [4]. Lysosomal accumulation of cystine, which is the hallmark of this autosomal recessive disorder, can be depleted by the amino-thiol cysteamine [5]. Although treatment with cysteamine substantially decreases intracellular cystine accumulation, renal Fanconi syndrome is not cured, but end stage renal disease can be postponed in the majority of the patients. On the other hand, recent findings in a ctns−/− mouse model suggest that cystine accumulation itself is not sufficient for the development of renal aberrations [6]. Several mechanisms have been postulated to link lysosomal cystine accumulation in cystinosis with renal tubular defects, such as impaired ATP synthesis [7,8], involvement of altered glutathione (GSH) metabolism [9,10] and increased apoptosis rate [11,12].

Decreased levels of total GSH in cystinotic fibroblasts during exponential growth were first reported by Choi et al. [9]. This finding was confirmed in primary proximal tubular cells derived from urine [13]. In contrast, normal total GSH levels but increased oxidized GSH (GSSG) levels have been reported in cystinotic fibroblasts grown to
confluence, in polymorphonuclear cells and in proximal tubular cells immunolabeled with HPV E6/E7 [10,14,15]. Together with the finding of elevated urinary 5-oxoproline (pyrroglutamic acid), a precursor for GSH synthesis, these data suggested that impaired lysosomal cystine efflux in cystinosis affects GSH metabolism, likely via impairment of γ-glutamyl-cycle [16]. Alterations in GSSG/2GSH ratio affect intracellular metabolic functions and point towards increased intracellular oxidation [17].

In addition to alterations in ATP-dependent GSH synthesis, decreased intracellular ATP levels have been reported in cells loaded with cystine dimethylster (CDME) [7]. Coor et al. used this model to postulate that decreased ATP synthesis accounts for decreased sodium gradient and, consequently, influences tubular reabsorption in cystinosis. Despite the limitations of the CDME model [18], this hypothesis is still pending, since decreased ATP levels in cystinotic fibroblasts and proximal tubule cells were reported [8,13]. More recently, decreased ATP generation capacity was shown in cystinotic renal proximal tubular cells [19].

Despite extensive research, definite conclusions whether alterations in GSH and/or ATP status are involved in the pathogenesis of cystinosis are lacking. In addition, the question remains why cysteamine treatment postpones the progression of renal disease and has no curative effect on Fanconi syndrome, despite the decrease in cystine levels. Likely, the anti-oxidative effect of cysteamine plays a role in this process, as cysteamine was demonstrated to increase GSH levels in mouse fibroblasts [20]. This study focuses for the first time on the influence of cysteamine on both GSH and ATP metabolism in vitro using a recently developed conditionally immortalized proximal tubular epithelial cell line (ciPTEC) [21,22]. Here, 4 control and 10 cystinotic ciPTEC lines with different mutations in the CTNS gene were developed from healthy controls and patients, using identical methodology. This allowed a valuable comparison of their metabolic status in presence and absence of cysteamine to improve our insight in the mechanism of cysteamine efficacy in cystinosis.

2. Materials and methods

2.1. Cell culture

Primary cell lines were cultured from urine of 4 healthy controls (age 60–152 months old) and 10 patients with cystinosis (age 11–209 months old) after approval of the study design by the Institutional Review Board and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects (age 11–209 months old) after approval of the study design by the Institutional Review Board and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22]. Diagnosis of cystinosis was made by measuring and obtaining written informed consent by the parents of all subjects as described previously [22].

To compare cell proliferation rate between control ($n = 4$) and cystinotic ($n = 6$) cell lines, cells were counted at several stages up to 10 days of maturation at 37 °C. Data are expressed as percentage increase in cell number when transferred from 33 to 37 °C. Additionally, total intracellular protein content was measured using Biorad Protein assay (Biorad Laboratories, Germany) during maturation of cells cultured in 24 well plates. Protein synthesis was determined at day 10 of maturation by measuring $[^{3}H]$-Leucine ([$^{3}H$]-Leu) incorporation. Control and cystinotic ciPTEC were cultured in triplo in 24 well plates in the presence of $[^{3}H]$-Leu (3 μCi/ml; GE Healthcare, UK) supplemented tissue culture medium for 2, 6, 16 and 24 h. Cells were washed using ice-cold PBS and proteins were precipitated using 10% (w/v) trichloroacetic acid. Proteins were dissolved in 400 μl NaOH (0.3 M) and subsequently to neutralization with 80 μl HCl (1.5 M) radioactivity was measured using a TriCarb liquid scintillation counter (Perkin Elmer, USA). Using specific activity of $[^{3}H]$-Leu, incorporation of Leu was calculated and expressed as pmol/mg protein. Cell cycle analysis was performed on proliferating and matured controls (control $n = 3$; cystinosis $n = 6$), by harvesting cells using trypsin and fixation in 70% (v/v) ice-cold ethanol. Subsequently, cells were stained using propidium iodide (10 μg/ml) in citrate buffer (0.2 M Na2HPO4, 0.1 M citric acid) in the presence of RNase (5 μg/ml). Cell cycle was determined by flow cytometry and presented as percentage of the cells in the S-phase of mitosis.

2.3. Albumin uptake in ciPTEC

Albumin uptake was performed as previously described [22] by analyzing BSA-FITC uptake by flow cytometry. Briefly, ciPTEC of controls ($n = 4$) and cystinotic patients ($n = 7$) were incubated for 30 min with 50 μg/ml BSA-FITC. Mean fluorescence intensity was maintained as mean ± SEM. To confirm that albumin uptake was megalin dependent, we performed receptor associated protein (RAP) inhibition assays on BSA-FITC uptake. RAP was kindly provided to us by Dr. M. Nielsen (University of Aarhus, Denmark).

2.4. Thiols and disulfide determination

Matured ciPTEC were harvested using trypsin/EDTA, washed twice in PBS and pellets were shock frozen in liquid nitrogen and stored at −80 °C until further processing. Total intracellular content of cysteine, cystine, GSH and GSSG was measured using HPLC as described before [10]. Cystine levels were measured in all control ($n = 4$) and cystinotic ($n = 10$) ciPTEC after maturation for 10 days at 37 °C. To test the effect of cysteamine on intracellular cystine levels, we performed a series of experiments varying its concentration (0.2; 1; 2 mM) and time of incubation (0.5; 1; 2; 4; 8; 16; 24; 48 h). To study the effect of long-term cysteamine treatment (1 mM) on intracellular thiol and disulfide levels, control ($n = 4$) and cystinotic ($n = 7$) ciPTEC were incubated in the

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age* (months)</th>
<th>Sex</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cys1</td>
<td>78</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
<tr>
<td>cys2</td>
<td>129</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
<tr>
<td>cys3</td>
<td>134</td>
<td>f</td>
<td>[c.141–24T→C] + [c.141–24T→C]</td>
</tr>
<tr>
<td>cys4</td>
<td>50</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
<tr>
<td>cys5</td>
<td>47</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
<tr>
<td>cys6</td>
<td>174</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
<tr>
<td>cys7</td>
<td>11</td>
<td>f</td>
<td>[57 kb del] + [c.927,928insG]</td>
</tr>
<tr>
<td>cys8</td>
<td>78</td>
<td>f</td>
<td>[57 kb del] + [c.6018,2GACT]</td>
</tr>
<tr>
<td>cys9</td>
<td>96</td>
<td>f</td>
<td>[57 kb del] + [c.665A→G]</td>
</tr>
<tr>
<td>cys10</td>
<td>20</td>
<td>m</td>
<td>[57 kb del] + [57 kb del]</td>
</tr>
</tbody>
</table>

m, male; f, female.

* Age in months at collection of urine.

8 From Ref. [49].
presence of 1 mM cysteamine for 48 h during which cysteamine supplemented culture medium was refreshed every 6 h. Data are expressed as nmol thiol/mg protein (±SEM). Furthermore, correlations between intracellular cystine and GSSG levels were determined.

2.5. Redox status calculation

Redox status (Eo) of GSSG/GSH pool was calculated using the Nernst equation according to Schafer and Buettner [17]: (Eo (mV) = (RT/nF) log ([GSH]/[GSSG]), where E\text{0} for pH 7.4 = −264 mV; RT/nF = 30 and concentrations for GSH and GSSG in M are calculated assuming that 1 mg protein corresponds to a cellular volume of 5 μl [25].

2.6. Determination of ROS and oxidation status

Possible alterations in reactive oxygen species (ROS) formation in cystinotic ciPTEC were analyzed using the probes 5- (and-6)-chloro-1-methyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-\text{H}_2\text{DCFDA}) and hydroethidine (HEt) [26]. Measurements were performed in matured controls (n = 4) and cystinotic (n = 10) ciPTEC.

The production of ROS using CM-\text{H}_2\text{DCFDA} was detected in ciPTEC using a 96 well assay [27]. Matured ciPTEC were washed in HEPES–Tris buffer (NaCl (132 mM), KCl (4.2 mM), CaCl\text{2} (1 mM), MgCl\text{2} (1 mM), D-glucose (5.5 mM), Heps (10 mM), pH 7.4 using Tris) and incubated for 15 min in the dark with 80 μl CM-\text{H}_2\text{DCFDA} (10 μM) Esterase hydrolyzes CM-\text{H}_2\text{DCFDA} to form intracellular trapped CM-\text{H}_2\text{DCF}, which can oxidize to fluorescent CM-DCF, e.g. by the action of ROS. Cells were washed twice in HEPES–Tris buffer and incubated in the absence and presence of H2O\text{2} (0; 10; 100 and 1000 μM) as a positive control to stimulate oxidation and to verify the sensitivity of the assay. Formation of CM-DCF as a marker for oxidation was monitored immediately and monitored for 15 min on Victor3 multilabel counter (ex464 nm, em530 nm; Wallac, Perkin Elmer). Additionally, one set of samples was pre-incubated with 1 mM cysteamine for 2.5 h at 37 °C. Basal and H2O\text{2} stimulated CM-\text{H}_2\text{DCF} oxidation was calculated as the slope of fluorescence intensity increase versus time and expressed as oxidation rate (±SEM) with untreated cells set at 100% after correction for protein content.

Alternatively, matured ciPTEC were loaded with HEt (10 μM) in HEPES–Tris buffer for 15 min in 24 well plates in presence or absence of the positive control rotenone (100 nM), Rotenone stimulates superoxide production by inhibiting complex I of the oxidative phosphorylation system [28]. The fluorescent oxidation products of non-fluorescent HEt, 2-hydroxyethidium and ethidium, were detected in ciPTEC using flow cytometry after harvesting the cells using trypsin/EDTA and washing in PBS. Additionally, one set of samples was pre-incubated with 1 mM cysteamine (1 mM for 2.5 h in culture medium. Data are expressed as mean fluorescence intensity (±SEM).

Possible changes in protein oxidation and lipid peroxidation were investigated by determining the amount of butylated protein and F2-isoprostane, respectively. Oxidative stress can lead to the introduction of carbonyl groups in proteins, detectable by immunoblotting using the Oxyblot™ protein oxidation detection kit (Millipore, USA). Matured ciPTEC of 4 controls and 7 patients were examined according to the manufacturer’s protocol. After immunoblotting and development using ECL Western blotting substrate (Thermo Fisher Scientific, USA), pixel intensity for each cell line was analyzed using ImagePro™ software.

Intracellular phosphate levels were determined in duplo in cell pellets of matured controls (n = 3) and cystinosis (n = 6) ciPTEC to test whether decreased ATP levels could be a result of decreased phosphate availability. Data are expressed as mM phosphate/mg protein ±SEM. Furthermore, alterations in sodium-dependent phosphate uptake in cystinosis ciPTEC were investigated using radio-labeled 32PO \text{4} as describe before [22]. Km and Vmax values were determined using phosphate saturation curves in control (n = 4) and cystinotic (n = 5) ciPTEC with GraphPad Prism (version 5.03, GraphPad Software Inc.). Further, experiments were performed with a pre-incubation of cysteamine (1 mM) for 2 h, followed by the uptake of K\text{2}HPO\text{4} (0.22 mM) in presence or absence of cysteamine.

2.9. Statistical analysis

To compare cystinosis and control ciPTEC and the effect of cysteamine, appropriate unpaired or paired Student's t-test were used for statistical analysis. Correlations were analyzed using Spearman test.
3. Results

3.1. Cell culture and proliferation

Primary cell lines derived from urine were successfully transfected using SV40T and hTERT vectors and subcloned as described before [22]. This resulted in 14 ciPTEC clones, derived from 4 individual control subjects and 10 cystinosis patients (Table 1). The presence of alkaline phosphatase activity confirmed proximal tubular origin and was similar in control and cystinosis cell lines (activity in control 2.5 ± 1.6 vs. cystinosis 2.3 ± 1.2 mU/mg protein). Additionally, the expression of the proximal tubular cell markers aminopeptidase N, aquaporin1, P-glycoprotein and dipeptidyl peptidase IV were similar in all cell lines (data not shown). Transfection with SV40T resulted in cell lines proliferating at 33 °C, while the disappearance of the SV40T antigen expression at 37 °C during 10 days resulted in cell maturation. The additional transfection using hTERT prevented the occurrence of replicative senescence. Proliferation of control and cystinosis ciPTEC was monitored for 10 days at 37 °C by cell count and protein determination. Both control and cystinosis ciPTEC proliferated similarly up to ~3 days (Fig. 1A). At this stage, SV40T expression was decreased as previously shown [22]. The total cell population was doubled at the end of the maturation period in both cell populations. Intracellular protein content of both control and cystinosis ciPTEC reached a plateau at day 6 of maturation (Fig. 1B). Both control and cystinosis ciPTEC readily incorporated 3H-Leu between days 8 and 10 of maturation, indicating the absence of any difference in protein synthesis (Fig. 1C). Cell cycle analysis using propidium iodide staining indicated that control and cystinosis cells had similar stages of cell division. At 33 °C, 11.0% (±0.5) and 68.3% (±2.3) of control ciPTEC compared to 12.8% (±2.5) and 64.3% (±5.6) of cystinosis ciPTEC were in the S-phase or G1/G0 phase respectively. After 10 days of maturation at 37 °C the percentage of cells in S-phase was decreased to 73.7% (±5.9) for control ciPTEC and 70.7% (±5.5) for cystinosis cells. No difference was observed between cystinosis and control cells. Further, no significant differences in cell division rate nor in intracellular protein content or incorporation were observed between control and cystinosis ciPTEC, allowing the expression of metabolic measurements as a function of protein content in further assays.

3.2. Albumin uptake in ciPTEC

Albumin uptake in both control and cystinotic ciPTEC was decreased by megalin-ligand RAP in a concentration dependent manner, suggesting endocytic uptake of albumin via megalin (Fig. 2A). To test whether albumin uptake was affected in cystinotic ciPTEC, we performed flow cytometry analysis of BSA-FITC uptake in 4 control cell lines and 7 cystinotic cell lines in two independent experiments in triplo (Fig. 2B). Both experiments demonstrated that albumin uptake in cystinotic cell lines was comparable to control ciPTEC (p = 0.75).

3.3. Intracellular thiol and disulfide content

Intracellular cystine levels in matured ciPTEC were significantly increased in the cystinosis ciPTEC clones derived from 10 cystinosis patients compared to 4 control ciPTEC (5.2 ± 0.7 versus 0.14 ± 0.02, respectively; p < 0.01; Fig. 3A). Each point represents one patient or control cell line, measured at least in 2 separate assays. The dose-dependent effect of cysteamine on intracellular cystine levels in cystinosis cell lines was determined, demonstrating that 1 mM cysteamine normalized the cystine content within 30 min after addition and for a period up to 8 h (data not shown). By refreshing the cysteamine supplemented culture medium every 6 h during 48 h, cystine levels in cystinosis ciPTEC were maintained in the control range (0.26 ± 0.07, p = 0.23; Fig. 3B). Free cysteine levels were also significantly increased in cystinosis ciPTEC (7.2 ± 1.2 versus 2.4 ± 0.5, p < 0.05). Similarly, free cysteine levels were normalized upon cysteamine treatment (2.1 ± 0.5, p = 0.87; Fig. 4A).

Total GSH levels were similar in cystinosis and control ciPTEC (34 ± 5 and 35 ± 6, respectively, p = 0.86; Fig. 4B). Remarkably, due to cysteamine treatment for 2 days, total GSH levels were significantly...
increased in both control (63 ± 10, p < 0.05) and cystinosis (95 ± 19; p < 0.01) ciPTEC, suggesting that cysteamine treatment increased cellular capacity to deal with oxidative stress (Fig. 4B).

GSSG levels were significantly increased in cystinosis cells (0.15 ± 0.04 versus 0.66 ± 0.16; p < 0.05) and correlated with intracellular cystine levels (p = 0.01; r² = 0.76; Fig. 4C). Cysteamine treatment tended to increase GSSG in both control and cystinosis ciPTEC, however this effect was not statistically significant in cystinosis cells. Using the Nernst equation according to Schäfer and Buettner [17], we estimated the redox effects in control and cystinosis cells with 1 mM cysteamine for 2 days (cysteamine).

3.4. Intracellular ROS production and oxidation status

To get an indication whether increased redox status was a result of increased in ROS production, oxidation was measured under basal and stressed conditions using the probes CM-H₂DCFDA and HEt. Oxidation of CM-H₂DCF to CM-DCF occurred linear in time and dose-dependently increased upon stimulation with H₂O₂ (data not shown). Basal oxidation of CM-H₂DCF did not differ between control and cystinosis ciPTEC (fluorescence intensities of 2515 ± 97 and 1988 ± 381, respectively; p = 0.40; Fig. 5A). H₂O₂ tended to increase the formation of CM-DCF in cystinosis cells compared to control cell lines, reaching statistical significance at 1000 μM (p < 0.05; Fig. 5B). Treatment of control and cystinosis cells with 1 mM cysteamine for 2.5 h did not alter basal and/or H₂O₂-stimulated CM-H₂DCF oxidation (data not shown).

No significant alterations in HEt oxidation were observed in control versus cystinosis ciPTEC (1.3 ± 0.4 versus 3.4 (± 1.1), p = 0.27; Fig. 5C). The addition of rotenone, a potent stimulator for mitochondrial ROS production, caused a significant increase in HEt oxidation levels, indicating that cells were not in a maximum state of oxidation (p < 0.05). Cysteamine treatment for 2.5 h did not alter HEt oxidation in control and/or cystinosis cells (data not shown). Using the Oxylight™ protein oxidation detection kit, we investigated possible changes in protein oxidation between control and cystinosis ciPTEC. However, no differences were detected (intensity values of 0.67 ± 0.05 and 0.82 ± 0.07, respectively; p = 0.19). In addition, IPF2α-VI, one of the major F2-isoprostanes, was determined as a marker for fatty acid oxidation. Levels of IPF2α-VI were comparable in control (0.33 ± 0.1) and cystinosis ciPTEC (0.35 ± 0.1; p = 0.79).

3.5. ATP metabolism

Intracellular ATP levels were measured in 21 characterized clones of the 4 control matured ciPTEC and 50 clones of the 10 cystinosis ciPTEC. ATP levels were significantly decreased in cystinosis ciPTEC (2.25 ± 0.36 versus 1.35 ± 0.12; p < 0.01), indicating alterations in ATP metabolism (Fig. 6A). Although in 5 cystinosis ciPTEC there was a trend towards an increase in intracellular ATP after 48 h of cysteamine treatment, this increase did not reach statistical significance (p = 0.08; Fig. 6B). To determine whether decreased ATP levels were a result of reduced ATP production, glycolysis and oxidative phosphorylation were investigated in intact matured cells expressing either cytosolic or mitochondrial targeted luciferase. Neither cytosolic (glycolysis) nor mitochondrial (oxidative phosphorylation) ATP production was altered in cystinosis cells (p values of 0.18 and 0.60, respectively; Fig. 6C). Using glycolysis inhibitors DOG and SIA, ATP levels were decreased (SIA: > 60%; DOG: > 90%) in one control and two cystinosis cell lines, indicating ciPTEC derived their ATP mainly from glycolysis. Since cultured ciPTEC are mainly glycolytic, we further explored the glycolytic activity. Both inhibitors significantly decreased the production of pyruvate in control (DOG: p < 0.05; SIA: p < 0.05) and cystinosis (DOG: p < 0.05; SIA: p < 0.01) ciPTEC. Pyruvate production was similar in control and cystinosis cells (Fig. 6D).

Next, we addressed the possibility that decreased ATP levels could be caused by a decreased phosphate availability. Measurement of intracellular phosphate revealed the absence of any difference between control and cystinosis ciPTEC (0.22 ± 0.04 versus 0.18 ± 0.01, respectively; p = 0.18). Additionally, sodium-dependent phosphate uptake was determined in ciPTEC, using radio-labeled 32PO₄ demonstrating decreased phosphate uptake in cystinotic cells (p < 0.05). By analyzing
the saturation curves of phosphate uptake in control and cystinotic cells, a $V_{\text{max}}$ (104.2±15.3 for control and 86.0±5.1 for cystinotic ciPTEC) and $K_m$ (0.15±0.08 and 0.20±0.04, respectively) could be determined (Fig. 7A). The presence of cysteamine (1mM) did not normalize phosphate uptake in cystinotic ciPTEC, despite the normalization of cystine levels (Fig. 7B).

4. Discussion and conclusions

In this study, 4 control and 10 cystinosis conditionally immortalized human cell lines were developed from urine of age matched donors using exfoliated cells of proximal tubular origin. Using this model, altered GSH redox status and decreased ATP levels were observed. Moreover, treatment with the cystine depleting agent cysteamine increased intracellular total GSH levels and restored the GSH redox status of the cystinosis cells, but did not normalize affected sodium-dependent phosphate uptake.

The advantage of the presented cell model of cystinosis with different mutations in CTNS gene is sufficient cystine accumulation, which is ~37-fold higher compared to control cells, approaching the levels measured in renal tissue [3]. Furthermore, cystine levels in cystinosis ciPTEC are approximately 6-fold higher compared to a previously reported cystinosis PTEC, immortalized using HPV 16 E6/E7 genes [15]. Probably, the maturation at 37 °C, causing decreased cell proliferation, allowed the cells to accumulate more cystine over time. As cells matured for 10 days, continued metabolism and lysosomal protein digestion could result in more cystine trapped in the lysosomes. The expression of the temperature sensitive vector SV40T strongly decreased at 37 °C allowing cells to differentiate. As a result, the metabolism of the cells was not (or was less) influenced by the presence of this viral antigen compared to the other cell models.

Fig. 5. Intracellular ROS production in ciPTEC. (A) Basic CM-H$_2$DCFDA oxidation is not altered in cystinotic ciPTEC. Each point represents a separate cell line of control (n=4) or cystinotic (n=8) donors, measured in triplo. (B) Control and cystinotic ciPTEC are both sensitive to H$_2$O$_2$-stimulated oxidation of CM-H$_2$DCF. At high levels of H$_2$O$_2$, oxidation was significantly increased in cystinotic cell lines (p values of 0.24; 0.11 and 0.05 for 10; 100 or 1000 μM H$_2$O$_2$, respectively. (C) Flow cytometric analysis of hydroethidine (HEI) oxidation, showing the absence of any statistically significant difference between control and cystinotic ciPTEC. Each point represents a separate cell line of control (n=4) or cystinotic (n=10) donors, measured in triplo. Gray dots represent cystinotic patients with a homozygous 57 kb deletion.

Fig. 6. ATP metabolism in ciPTEC. (A) Intracellular levels of ATP are significantly decreased cystinotic ciPTEC. (B) Treatment of cystinotic ciPTEC with 1 mM cysteamine for 48 h tends to increase intracellular ATP (p=0.08). (C) Measurement of cytosolic and mitochondrial ATP production using cytosolic and mitochondrial targeted luciferase, respectively. During luciferin perfusion, ATP-dependent luminescence is monitored as a measure of ATP production. Cystinotic ciPTEC display normal cytosolic and mitochondrial ATP production. (D) Pyruvate production as a measure of cytosolic ATP production in matured ciPTEC incubated in the absence or presence of deoxyglucose (DOG) and sodium iodo-acetate (SIA), two potent inhibitors of glycolysis. Both inhibitors significantly decrease glycolytic activity to the same extent in control and cystinotic ciPTEC. Black bars, control; white bars, cystinosis *p<0.05.

Fig. 7. Sodium-dependent phosphate uptake in ciPTEC. (A) Phosphate saturation curves in control (n=4; continuous line) and cystinotic (n=5; dashed line) ciPTEC, demonstrating decreased phosphate reabsorption in cystinosis (p=0.05). (B) Uptake of $^{32}$PO$_4^{2-}$ was measured in the presence or absence of cysteamine (1 mM). Cysteamine treatment has no effect on $^{32}$PO$_4^{2-}$ uptake in control and cystinotic cells. Black bars, control; white bars, cystinosis.
Additionally, in this study both cystinosis and control cell lines were established using the same methodology from pediatric donors, after similar passage number and in identical phases of the cell cycle, allowing a valuable comparison of their metabolic status. As the study population comprises ciPTEC derived from cystinosis patients with homozygous 57 kb deletion and other mutations in the CTNS gene, a genotype–phenotype correlation could be further investigated. However, we neither observed differences in cystine levels nor in ROS production between the two groups of cells. This is coherent with the indistinguishable renal phenotype observed in patients having a homozygous 57 kb deletion compared to other truncating mutations.

In the present study, the effect of cysteamine on GSH status was studied for the first time in a renal cystinosis cell model. Remarkably, total levels of GSH increased and the GSH redox status normalized upon cysteamine treatment. In addition to depleting cystine levels, this indicates that cysteamine acts as an antioxidant, hence, is a protective agent against oxidative stress. Next to cystine depletion, this mechanism may underlie the protective action of cysteamine against the development of interstitial fibrosis, which leads to end stage renal disease in cystinosis. In line with our findings, Chol et al. [9] described a trend towards increased levels in GSH upon incubation with cysteamine, ornithine and N-acetyl-cysteine. As cysteamine restores the GSH redox status, this agent may have beneficial effects in other disorders associated with increased peroxidation, as oxidative damage has been proposed to be involved in the pathogenesis in chronic kidney disease [32,33]. Recently, the use of cysteamine has also been proven to have a synergistic effect on the anti-malarial activity of artemisinin-derivatives, implicating the broader use of this agent [34].

It has been suggested that limited cysteine availability due to dysfunctional cystine efflux from lysosomes in cystinosis was responsible for decreased GSH synthesis via the γ-glutamyl cycle [10]. Contrasting this assumption, we and others demonstrated increased intracellular levels of cystine indicating that limited cysteine availability is not the cause for alterations in γ-glutamyl cycle [35,36]. Elevated free cysteine levels in cystinosis cells can derive from the extracellular medium or synthesis from methionine. A recent study by Bellomo et al. showed that intracellular cysteine levels regulate the CTNS gene expression [35]. Silencing of the CTNS gene was associated with an increase in intracellular cysteine comparable to our results in cystinosis ciPTEC. Interestingly, Foreman et al. demonstrated increased cysteine in CDME loaded rats, which developed renal Fanconi syndrome, without elevated levels of cystine [37]. The mechanism of elevated cysteine in cystinosis proximal tubular cells should be investigated further.

The formation of GSH from lysosomally accumulated cystine upon cysteamine treatment can only partially explain elevated GSH levels, regarding the stoichiometry. Free intracellular cysteine (7.2 mol/mg protein) and intracellular cysteine (equal to 2 × 5.2 mol/mg protein = 10.4 mol cysteine/mg protein) in cystinosis ciPTEC are 3.5-fold lower than required for the increase of GSH (95–34 = 61 mol cysteine/mg protein) upon cysteamine treatment. Furthermore, proximal tubular GSH transporters might be activated by cysteamine, as tubular GSH levels in vivo are mainly dependent on uptake at the apical and basolateral membrane via specific transporters [38,39].

In the cystinosis ciPTEC model, the GSH pool is still sufficient to protect proteins and fatty acids from oxidative damage at basal conditions as shown in this study. However, when cystinotic cells are exposed to high levels of oxidation, increased ROS generation is observed. A similar situation can occur in vivo, because of the active oxidative metabolism in renal proximal tubules, making cystinosis cells prone to the oxidative stress. Further, in vivo increased protein concentration in the tubular lumen can exaggerate oxidative stress leading to progressive tubulo-interstitial inflammation [40]. In this situation, intracellular GSH availability can be of significant importance and, therefore, optimal cysteamine treatment protecting against peroxidation can improve renal survival. In addition, due to the prominent role of the mitochondrial ATP production, an increased ROS production compared to the in vitro model can be expected requiring more GSH for protection. Hence, alterations in GSH metabolism may induce apoptotic cell death and cause mitochondrial oxidative damage [41,42]. Therefore, the various hypotheses with involvement of GSH metabolism, ATP metabolism or enhanced cell death may represent different facets of a unique cascade leading to tubular dysfunction initiated by lysosomal cystine accumulation [11,19].

In contrast to our results, a recent study in fibroblasts showed intact redox status as presented by GSSG:GSH ratio, with normal total GSH levels [36]. The latter study correctly pointed towards the difficulties in measuring disulfides and mentioned a possible underestimation of the total GSH pool in the fibroblasts previously measured by our group [10]. Taken into account that 1 mg protein corresponds to a cell volume of 5 μl [25], the levels measured in matured control ciPTEC are 35 nmol GSH/mg protein/5 μl and equals to 7 mM. Hence, GSH levels measured in cultured ciPTEC in the current study are comparable to the levels measured in tubular cells in vivo, which are reported to be in the range of 5 mM [38]. Furthermore, in the current study, the cystine pool is about 10-fold lower than the GSH pool in control ciPTEC, which is comparable to other studies [35,36]. The observed variability in the intracellular thiol concentration between individual cell lines points to the necessity to analyze sufficient number of cell lines for making statistically valuable comparisons.

In our study, decreased levels of intracellular ATP in cystinosis ciPTEC were observed. Both glycolytic and mitochondrial ATP production were intact, suggesting that decreased ATP levels were a result of increased ATP consumption. A possible mechanism by which ATP consumption can be increased in cystinosis was recently suggested by Kumar et al. [43]. They proposed that a futile turnover of 5-oxoproline to glutamate in the absence (or decreased availability) of cysteine, consuming two additional ATP molecules per cycle, might be responsible for the decreased ATP and explain elevated excretion of 5-oxoproline into cystinosis urine. As we found no decreased levels of intracellular cysteine in cystinosis, this mechanism, in our opinion, is not explaining the observed decrease in ATP. Alternatively, a defect in the Na-dependent 5-oxoproline transporter in renal proximal tubules might be responsible for the increased excretion of this metabolite [44]. The artificial loading with CDME was recently used by Figueiredo et al. to demonstrate decreased adenylyl cyclase activity [45]. They suggested that cystine inhibition of this enzyme, catalyzing the phosphate transfer between ATP and AMP, could be partially responsible for the observed ATP decrease in cystinosis cells. In our previous study, we showed that cysteamine treatment did not improve cellular phosphate uptake despite slight increase of intracellular ATP levels, implicating that elevated cystine levels are not directly associated defective proximal tubular reabsorption. The latter observation corresponds to persistence of Fanconi syndrome in vivo in cystinosis patients despite cysteamine therapy [46]. Moreover, these findings demonstrate again that the CDME loading technique is of limited value in studying the pathogenesis of cystinosis [18].

Defective albumin uptake, another feature of Fanconi syndrome, could not be demonstrated in the cystinotic ciPTEC model. This is in line with our previous study demonstrating both intact apical expression of megalin together with abundant endocytic vesicles containing albumin and other megalin ligands in human cystinotic kidney tissue [47]. Thus, the origin of proteinuria can be different in cystinosis compared to other pathological conditions manifesting with renal Fanconi syndrome, such as Dent disease, in which apical expression of megalin in PTEC is decreased [48].

Taken together, the identical preparation of four control and ten cystinosis ciPTEC in the present study allows valid and detailed in vitro investigation of cell metabolism involved in the pathogenesis of cystinosis. Using this model, increased oxidation of GSH was demonstrated resulting in altered intracellular redox status in cystinosis cells. Cysteamine increased intracellular GSH levels and restored redox status of GSSG:GSH couple. This mechanism may be responsible for better
conservation of renal function in cystinosis patients treated with cysteamine. The increase of intracellular GSH in control cells upon cysteamine treatment points to the possibility of using this amino thiol in other renal disorders associated with enhanced oxidative stress.

Acknowledgements

The work presented in this study was performed thanks to financial support from Cystinosis Research Foundation, Cystinosis Research Network, funding from European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 201590 and Fund for Scientific Research, Flanders (F.W.O. Vlaanderen) [grant 18011N] The authors thank Henk Blom of VU Amsterdam and Sjoerd Verkaart of the Radboud University Nijmegen Medical Centre and Francesco Emma of the Bambino Gesù Children's Hospital Rome, Italy, for fruitful discussions. Measurement of intracellular phosphate levels under supervision of Prof. Hans Willems is appreciated. Technical assistance by Dinny van Oppenraaij-Emmerzaal, Jannene Keizer-Garritsen and Gowendolyn Beckmann is gratefully acknowledged.

References

