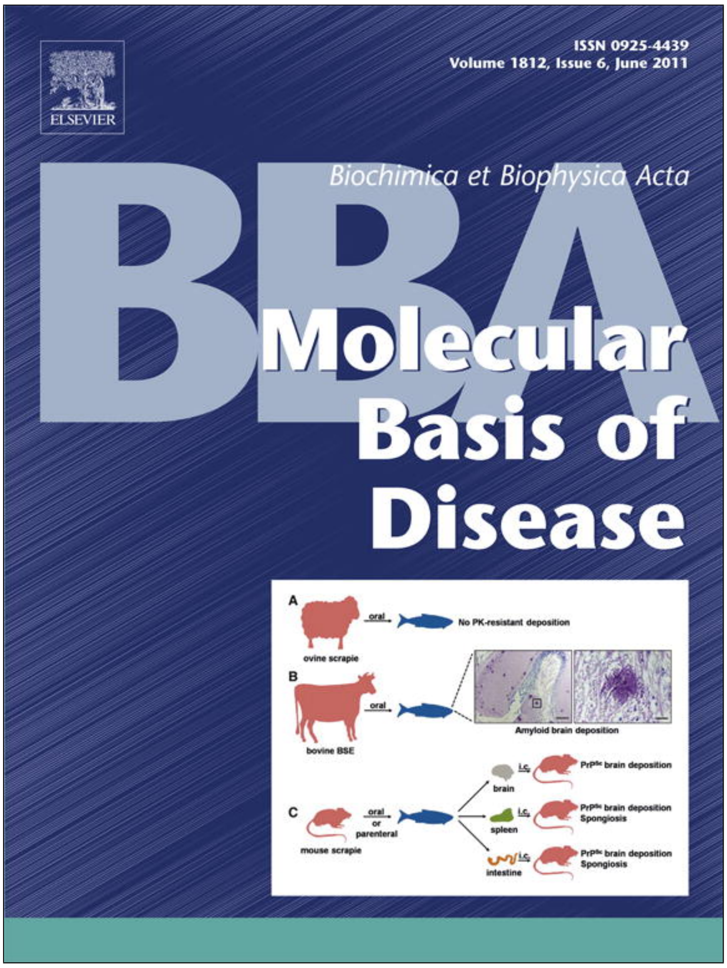

ISSN 0925-4439
Volume 1812, Issue 6, June 2011

ELSEVIER

Biochimica et Biophysica Acta

BBA Molecular Basis of Disease

Biochimica et Biophysica Acta 1812 (2011) 643–651

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

Martijn J. Wilmer [a,b,e], Leo A.J. Kluijtmans [a], Thea J. van der Velden [a], Peter H. Willems [c], Peter G. Scheffer [d], Rosalinde Masereeuw [e], Leo A. Monnens [f], Lambertus P. van den Heuvel [a,b], Elena N. Levtchenko [b,]*

[a] *Laboratory of Genetic Endocrine and Metabolic Diseases, Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, The Netherlands*
[b] *Department of Pediatrics, University Hospital, Leuven, Belgium*
[c] *Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, The Netherlands*
[d] *Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands*
[e] *Department of Pharmacology and Toxicology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, The Netherlands*
[f] *Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, The Netherlands*

ARTICLE INFO

Article history:
Received 20 June 2010
Received in revised form 28 January 2011
Accepted 22 February 2011
Available online 28 February 2011

Keywords:
Cystinosis
Cysteamine
Glutathione
Oxidative stress
Redox status
ATP production

ABSTRACT

Recent evidence implies that impaired metabolism of glutathione has a role in the pathogenesis of nephropathic cystinosis. This recessive inherited disorder 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.

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 the development of renal Fanconi syndrome in the first year of life and leads to end stage renal disease in the first decade of life when untreated [3]. 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 Chol 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

---

Abbreviations: CTNS, cystinosin; GSH, glutathione; GSSG, oxidized glutathione; CDME, cystine dimethylester; ciPTEC, conditionally immortalized proximal tubular epithelial cell line; DIG, digitonin; SV40T, SV40 ts A58; hTERT, human telomerase reverse transcriptase; ^{3}H-leu, ^{3}H-leucine; ROS, reactive oxygen species; HEt, hydroethidine; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; iPF2α-VI, 8-iso-prostaglandin F2α VI; DOG, deoxy-glucose; SIA, sodium iodoacetate

* Corresponding author at: Gasthuisberg Hospital Leuven, Department of Paediatrics, PO 7003, Herestraat 49, B-3000 Leuven, Belgium. Tel.: +32 16 341368.

E-mail address: elena.levtchenko@uz.kuleuven.ac.be (E.N. Levtchenko).

0925-4439/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2011.02.010

confluence, in polymorphonuclear cells and in proximal tubular cells immortalized with HPV E6/E7 [10,14,15]. Together with the finding of elevated urinary 5-oxoproline (pyroglutamic 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 dimethylester (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 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 elevated intracellular cystine levels in polymorphonuclear granulocytes and confirmed by molecular analysis of the *CTNS* gene (Table 1). Control cell lines were obtained from pediatric healthy volunteers with no clinical history of renal disease, nor with any other chronic disease. To maintain cell proliferation, primary cell lines were transfected with SV40T ts A58 (SV40T) and hTERT (human telomerase reverse transcriptase) followed by subcloning procedures to obtain homozygous cell populations [22–24]. Proximal tubular origin of all tested cell lines was confirmed as described previously [22] by demonstrating presence of aminopeptidase N, aquaporin1, P-glycoprotein and dipeptidyl peptidase IV using Western blotting. Additionally, sodium-dependent phosphate transport and alkaline phosphatase activity were analyzed. Of each donor, one subclone was selected on the basis of expression pattern and activity of the proximal tubular characteristics and used for further experiments, unless mentioned otherwise. In total, subcloned ciPTEC were used from 10 cystinotic patients and 4 healthy controls. Routinely, cells were maintained at permissive temperature of 33 °C (proliferating cells). To decrease expression of SV40T, cells were transferred to 37 °C and allowed to mature for up to 10 days (matured cells).

Table 1
Clinical information and *CTNS* mutation of the patients from whom ciPTEC were derived.

Cell line	Age[a] (months)	Sex	Mutation
cys1	78	m	[57 kb del] + [57 kb del]
cys2	129	m	[57 kb del] + [57 kb del]
cys3	134	f	[c. 141–24T>C] + [c. 141–24T>C][b]
cys4	50	m	[57 kb del] + [57 kb del]
cys5	47	m	[57 kb del] + [57 kb del]
cys6	174	m	[57 kb del] + [c.696_697insC]
cys7	11	f	[57 kb del] + [c.927_928insG]
cys8	78	f	[57 kb del] + [c.del18_21GACT]
cys9	96	f	[57 kb del] + [c.665A>G]
cys10	209	m	[57 kb del] + [57 kb del]

m, male; f, female.

[a] Age in months at collection of urine.

[b] From Ref. [49].

2.2. Cell proliferation

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 cells (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 Na_2HPO_4, 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 presented 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 trypsine/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 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 (E_h) of GSSG/2GSH pool was calculated using the Nernst equation according to Schafer and Buettner [17]:(E_h (mV) = $E_0 - RT/nF$ log ($[GSH]^2/[GSSG]$)),where E_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)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), and hydroethidine (HEt) [26]. Measurements were performed in matured control ($n = 4$) and cystinotic ($n = 10$) ciPTEC.

The production of ROS using CM-H_2DCFDA 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), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), D-glucose (5.5 mM), Hepes (10 mM), pH 7.4 using Tris) and incubated for 15 min in the dark with 80 µl CM-H_2DCFDA (10 µM). Esterase hydrolyzes CM-H_2DCFDA to form intracellular trapped CM-H_2DCF, 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 H_2O_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 Victor3V 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 H_2O_2 stimulated CM-H_2DCF 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 trypsine/EDTA and washing in PBS. Additionally, one set of samples was pre-incubated with 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 (MediaCybernetics) and corrected for GAPDH expression.

Intracellular concentrations of F2-isoprostanes (8-iso-prostaglandin F2α VI; iPF2 α-VI) were determined as marker of oxidative stress using LC-MS/MS. Briefly, butylated hydroxytoluene (0.08% (v/v)) was added to cell pellets to prevent auto-oxidation, and deuterated internal standard (0.2 ng iPF2α-d4, Cayman) was added to correct for loss during sample preparation. Then, the cells were sonicated for 3 × 10 s at 4 °C to lyse the cells. Afterwards, KOH was added (final concentration of 1.3 M) and the lysate was incubated for 1 h at 40 °C. Formic acid (20% (v/v)) was added to adjust to pH 4.5 and samples were prepared using solid-phase extraction as reported previously [29]. The eluate was dried under a stream of nitrogen at room temperature and redissolved in 100 µl acetonitrile (10% (w/v)). F2-isoprostanes were analyzed by a 4000 Qtrap (PE Sciex, Canada) MS. The concentration of iPF2α-VI was calculated using a standard curve of iPF2α-VI (Cayman) and corrected for amount of protein. Data are expressed as ng/mg protein.

2.7. ATP metabolism

Intracellular ATP levels were measured in control (21 separate clones of 4 donors) and cystinotic (50 separate clones of 10 donors) matured ciPTEC using ATP Bioluminescence Assay Kit HSII (Roche, Germany) as described before [15]. The effect of cysteamine on intracellular ATP levels was determined in cystinotic ciPTEC ($n = 4$) cultured in presence of cysteamine (1 mM) for 48 h, with medium replacement every 6 h.

Mitochondrial and cytosolic ATP production were measured by transfecting proliferating ciPTEC with baculovirus containing cDNA for mitochondria targeted luciferase or luciferase without targeting sequence as described before [30]. Briefly, proliferating ciPTEC (control $n = 2$, cystinosis $n = 2$; both measured in 4-fold) were cultured on coverslips and transfected for expression of cytosolic or mitochondrial luciferase. After two additional days of culturing at 37 °C, ATP production in intact ciPTEC was determined by perfusing the cells with 25 µM luciferin in Hepes–Tris buffer and measuring the luciferase luminescence using photomultiplier tube. After reaching a stable signal, cells were perfused with digitonin (DIG; 100 µM) and excess ATP (10 mM). Permeabilization of cellular membranes by DIG in the presence of excess ATP resulted in a peak in luminescence signal, which was limited by total luciferase present in the cells. Basal cytosolic and mitochondrial ATP production are expressed as percentage of the peak in luminescence after DIG treatment (±SEM).

Cytosolic ATP production from glycolysis was measured by determining pyruvate production in conditioned culture medium of ciPTEC and by measuring ATP levels in the presence of inhibitors of glycolysis [8,31]. Briefly, matured ciPTEC (control $n = 5$; cystinosis $n = 8$) were cultured in serum-free culture medium for 2.5 h in presence or absence of glycolysis inhibitors sodium iodoacetate (SIA; 0.02 mM) or deoxyglucose (DOG; 100 mM). Pyruvate production was subsequently measured in culture medium using lactate dehydrogenase (100 U/ml; Roche, Germany) after addition of NAD^+ (2 mM; Roche, Germany) at pH 9.5. Formation of NADH is directly correlated to glycolytic pyruvate production and was analyzed by measuring the absorbance at 340 nm on Victor3V Multilabel Counter (Wallac, Perking Elmer, USA).

2.8. Phosphate transport and content

Intracellular phosphate levels were determined in duplo in cell pellets of matured control ($n = 3$) 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 $^{32}PO_4$ as describe before [22]. K_m and V_{max} 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_2HPO_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 ^{3}H-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 3.0% (±0.8; $p<0.01$) and 5.0% (±0.7; $p<0.05$), respectively. The percentage of G1/G0 phase, indicative for differentiation, increased after maturation 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 ($p=0.75$).

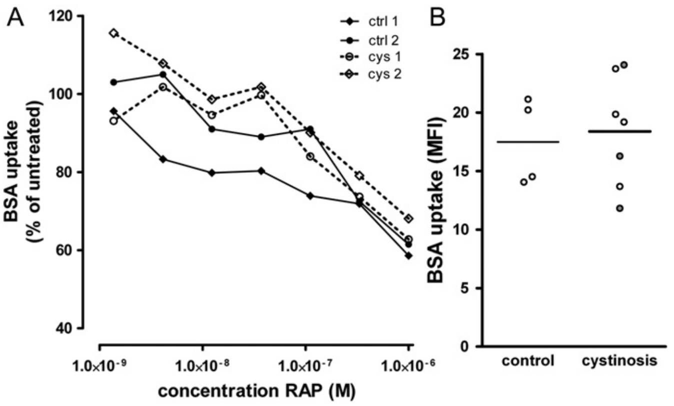

Fig. 2. Albumin uptake in ciPTEC. (A) Albumin uptake in both control and cystinotic ciPTEC was decreased by megalin-ligand RAP in a concentration dependent manner. (B) Flow cytometry analysis of BSA-FITC uptake in control cell lines ($n=4$) and cystinotic cell lines ($n=7$) demonstrated that albumin uptake in cystinotic cell lines was normal compared to control ciPTEC ($p=0.75$). Gray dots represent cystinotic patients with a homozygous 57 kb deletion.

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

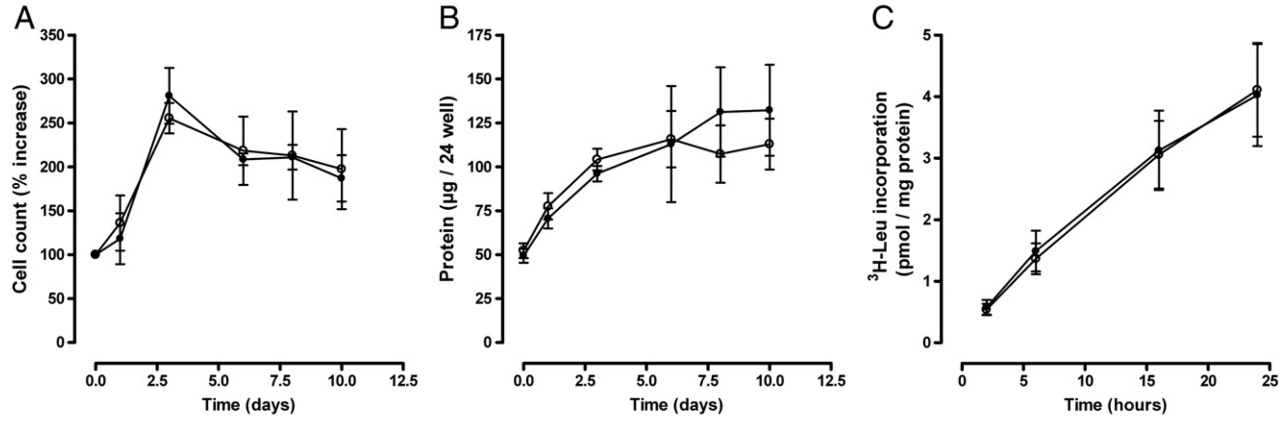

Fig. 1. Cell proliferation of control and cystinotic ciPTEC. (A) Cell proliferation decreases at approximately 3 days after raising the temperature to 37 °C to induce cell maturation. (B) Protein content as expressed per well plateaus at approximately 6 days after maturation induction. (C) Twenty-four hour incorporation of ^{3}H-Leu measured at day 9 of maturation. Matured control (closed circles) and cystinotic (open circles) ciPTEC show no differences in cell number and protein synthesis.

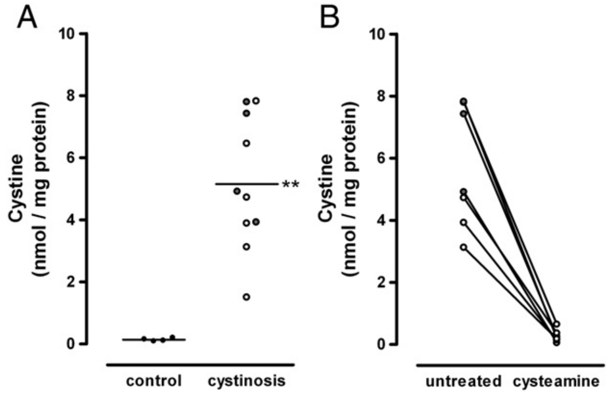

Fig. 3. Cystine accumulation in ciPTEC. (A) HPLC analysis reveals significantly elevated cystine levels in single clones from matured cystinotic ciPTEC derived from eight donors compared to control ciPTEC clones derived from four donors ($p<0.01$). (B) Treatment with 1 mM cysteamine for 2 days (replacement every 6 h), restores cystine levels in cystinotic ciPTEC. Gray dots represent cystinotic patients with a homozygous 57 kb deletion. **$p<0.01$.

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 ($p<0.01$; $r^2=0.76$; 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 Schafer and Buettner [17], we estimated the redox status of the GSSG/2GSH couple in ciPTEC. Our calculations revealed that the reduction potential was significantly less negative in matured cystinosis ciPTEC (−270 ± 4 versus −251 ± 1; $p<0.01$; Fig. 4D). The reduction potential in cystinosis ciPTEC was restored to control values upon cysteamine treatment (−269 ± 3; $p=0.43$).

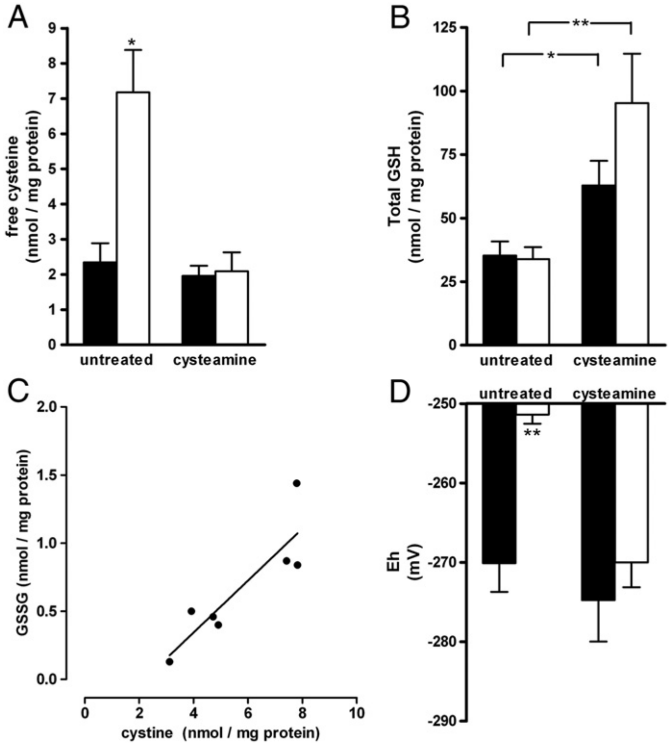

Fig. 4. Intracellular thiol levels in ciPTEC. (A) Free cysteine levels are increased in untreated cystinotic ciPTEC and decreased upon cysteamine treatment for 2 days (cysteamine). (B) Total GSH levels are similar in control and cystinotic ciPTEC and increased upon cysteamine treatment. (C) There is a significant correlation between intracellular cystine levels and GSSG levels in cystinotic ciPTEC, suggesting that lysosomal cystine accumulation influences intracellular GSH redox status ($p<0.01$; $r^2=0.76$). (D) Redox status (E_h (mV)), calculated using the Nernst equation, is less negative in cystinotic cells. Cysteamine treatment restores the redox status in cystinotic cells. Black bars, control; white bars, cystinosis. *$p<0.05$; **$p<0.01$.

3.4. Intracellular ROS production and oxidation status

To get an indication whether increased redox status was a result of increase in ROS production, oxidation was measured under basal and stressed conditions using the probes CM-H_2DCFDA and HEt. Oxidation of CM-H_2DCF to CM-DCF occurred linear in time and dose-dependently increased upon stimulation with H_2O_2 (data not shown). Basal oxidation of CM-H_2DCF did not differ between control and cystinosis ciPTEC (fluorescence intensities of 2515 ± 97 and 1988 ± 381, respectively; $p=0.40$; Fig. 5A). H_2O_2 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 H_2O_2-stimulated CM-H_2DCF 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 Oxyblot™ 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 iPF2a-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 mitochondria 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 derive 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 $^{32}PO_4$ demonstrating decreased phosphate uptake in cystinotic cells ($p<0.05$). By analyzing

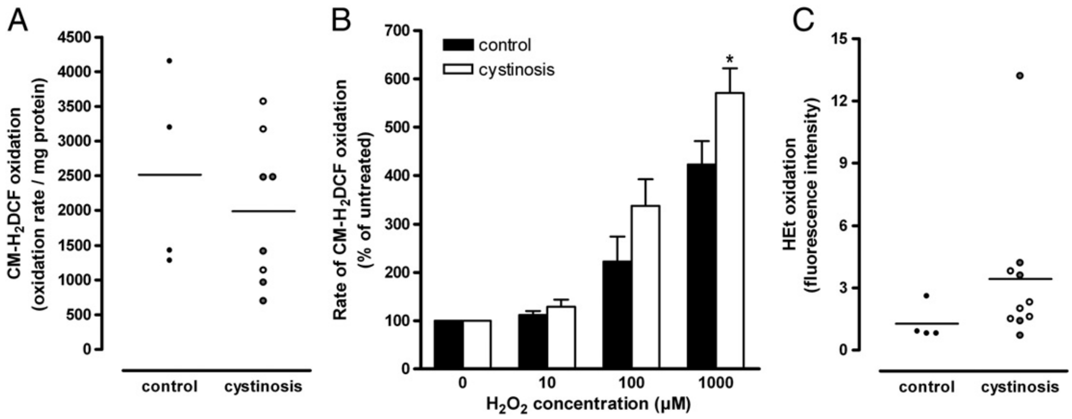

Fig. 5. Intracellular ROS production in ciPTEC. (A) Basic CM-H_2DCFDA oxidation is not altered in cystinotic ciPTEC. Each point represents a separate cell line of control ($n = 4$) or cystinotic ($n = 8$) donors, measured in triplo. (B) Control and cystinotic ciPTEC are both sensitive to H_2O_2-stimulated oxidation of CM-H_2DCF. At high levels of H_2O_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_2O_2, respectively. (C) Flow cytometric analysis of hydroethidine (HEt) 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.

the saturation curves of phosphate uptake in control and cystinotic cells, a V_{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).

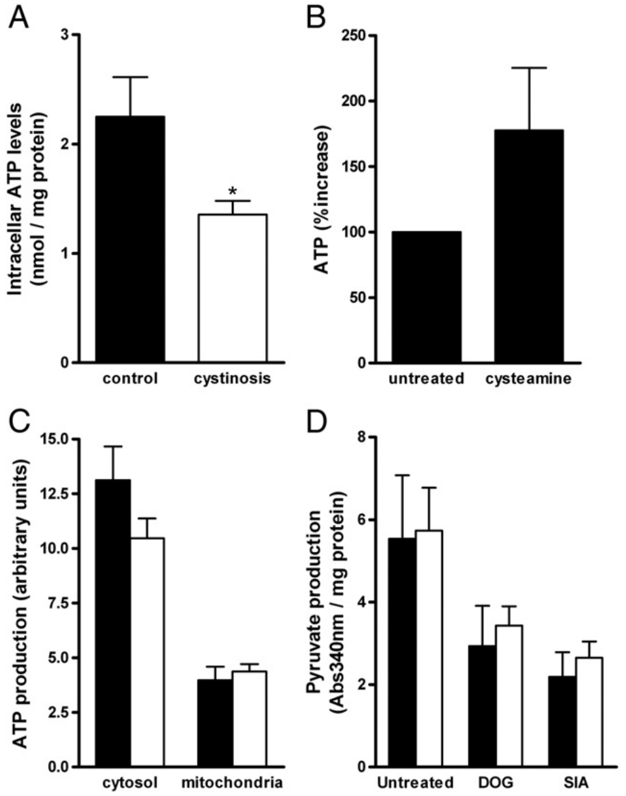

Fig. 6. ATP metabolism in ciPTEC. (A) Intracellular levels of ATP are significantly decreased cystinotic ciPTEC. (B) Treatment of cystinotic ciPTEC with 1 mM cysteamine for 48 h tends to increase intracellular ATP ($p = 0.08$). (C) Measurement of cytosolic and mitochondrial ATP production using cytosolic 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 *$p < 0.05$.

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.

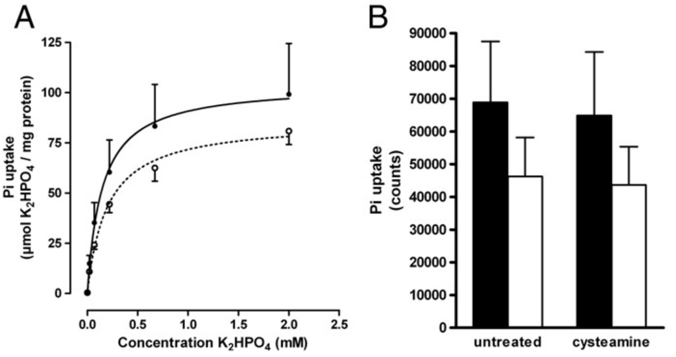

Fig. 7. Sodium-dependent phosphate uptake in ciPTEC. (A) Phosphate saturation curves in control ($n = 4$; continuous line) and cystinotic ($n = 5$; dashed line) ciPTEC, demonstrating decreased phosphate reabsorption in cystinosis ($p < 0.05$). (B) Uptake of $^{32}PO_4^{2-}$ was measured in the presence 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 γ-glutamyl cycle [10]. Contrasting this assumption, we and others demonstrated increased intracellular levels of cysteine 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 stochiometry. Free intracellular cysteine (7.2 mol/mg protein) and intracellular cystine (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/2GSH 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 cysteine 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 adenylate 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 present 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/2GSH 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 1801110N). 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, Jenneke Keizer-Garritsen and Gwendolyn Beckmann is gratefully acknowledged.

References

[1] M. Yudkoff, J.W. Foreman, S. Segal, Effects of cysteamine therapy in nephropathic cystinosis, N. Engl. J. Med. 304 (1981) 141.

[2] W.A. Gahl, Early oral cysteamine therapy for nephropathic cystinosis, Eur. J. Pediatr. 162 (2003) S38–S41.

[3] W.A. Gahl, J.G. Thoene, J.A. Schneider, Cystinosis: a disorder of lysosomal membrane transport, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The metabolic and molecular bases of inherited disease, McGraww-Hill, New York, 2001, pp. 5085–6108.

[4] M. Town, G. Jean, S. Cherqui, M. Attard, L. Forestier, S.A. Whitmore, D.F. Callen, O. Gribouval, M. Broyer, G.P. Bates, W. Van't Hoff, C. Antignac, A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis, Nat. Genet. 18 (1998) 319.

[5] W.A. Gahl, F. Tietze, J.D. Butler, J.D. Schulman, Cysteamine depletes cystinotic leucocyte granular fractions of cystine by the mechanism of disulphide interchange, Biochem. J. 228 (1985) 545.

[6] S. Cherqui, C. Sevin, G. Hamard, V. Kalatzis, M. Sich, M.O. Pequignot, K. Gogat, M. Abitbol, M. Broyer, M.C. Gubler, C. Antignac, Intralysosomal cystine accumulation in mice lacking cystinosin, the protein defective in cystinosis, Mol. Cell. Biol. 22 (2002) 7622.

[7] C. Coor, R.F. Salmon, R. Quigley, D. Marver, M. Baum, Role of adenosine-triphosphate (Atp) and Nak Atpase in the inhibition of proximal tubule transport with intracellular cystine loading, J. Clin. Investig. 87 (1991) 955.

[8] E.N. Levtchenko, M.J.G. Wilmer, A.J.M. Janssen, J.B. Koenderink, H.J. Visch, P.H.G.M. Willems, A. Graaf-Hess, H.J. Blom, L.P. Vandenheuvel, L.A. Monnens, Decreased intracellular ATP content and intact mitochondrial energy generating capacity in human cystinotic fibroblasts, Pediatr. Res. 59 (2006) 287.

[9] M. Chol, N. Nevo, S. Cherqui, C. Antignac, P. Rustin, Glutathione precursors replenish decreased glutathione pool in cystinotic cell lines, Biochem. Biophys. Res. Commun. 324 (2004) 231.

[10] E. Levtchenko, A. Graaf-Hess, M. Wilmer, L. van den Heuvel, L. Monnens, H. Blom, Altered status of glutathione and its metabolites in eystinotic cells, Nephrol. Dial. Transplant. 20 (2005) 1828.

[11] M.A. Park, V. Pejovic, K.G. Kerisit, S. Junius, J.G. Thoene, Increased apoptosis in cystinotic fibroblasts and renal proximal tubule epithelial cells results from cysteinylation of protein kinase Cdelta, J. Am. Soc. Nephrol. 17 (2006) 3167.

[12] P. Sansanwal, N. Kambham, M.M. Sarwal, Caspase-4 may play a role in loss of proximal tubules and renal injury in nephropathic cystinosis, Pediatr. Nephrol. (2009).

[13] G.F. Laube, V. Shah, V.C. Stewart, I.P. Hargreaves, M.R. Haq, S.J.R. Heales, W.G. van't Hoff, Glutathione depletion and increased apoptosis rate in human cystinotic proximal tubular cells, Pediatr. Nephrol. 21 (2006) 503.

[14] L. Mannucci, A. Pastore, C. Rizzo, F. Piemonte, G. Rizzoni, F. Emma, Impaired activity of the gamma-glutamyl cycle in nephropathic cystinosis fibroblasts, Pediatr. Res. 59 (2006) 332.

[15] M.J.G. Wilmer, A. Graaf-Hess, H.J. Blom, H.B.P.M. Dijkman, L.A. Monnens, L.P. van den Heuvel, E.N. Levtchenko, Elevated oxidized glutathione in cystinotic proximal tubular epithelial cells, Biochem. Biophys. Res. Commun. 337 (2005) 610.

[16] C. Rizzo, A. Ribes, A. Pastore, C. Dionisi-Vici, M. Greco, G. Rizzoni, G. Federici, Pyroglutamic aciduria and nephropathic cystinosis, J. Inherit. Metab. Dis. 22 (1999) 224.

[17] F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, Free Radic. Biol. Med. 30 (2001) 1191.

[18] M.J. Wilmer, P.H. Willems, S. Verkaart, H.J. Visch, D.E. Graaf-Hess, H.J. Blom, L.A. Monnens, L.P. van den Heuvel, E.N. Levtchenko, Cystine dimethylester model of cystinosis: still reliable? Pediatr. Res. 62 (2007) 151.

[19] P. Sansanwal, B. Yen, W.A. Gahl, Y. Ma, L. Ying, L.J. Wong, M.M. Sarwal, Mitochondrial autophagy promotes cellular injury in nephropathic cystinosis, J. Am. Soc. Nephrol. 21 (2010) 272.

[20] R. Djurhuus, A.M. Svardal, P.M. Ueland, Growth state dependent increase of glutathione by homocysteine and other thiols, and homocysteine formation in glutathione depleted mouse cell lines, Biochem. Pharmacol. 39 (1990) 421.

[21] L.C. Racusen, P.D. Wilson, P.A. Hartz, B.A. Fivush, C.R. Burrow, E.T. Philip, Renal proximal tubular epithelium from patients with nephropathic cystinosis—immortalized cell-lines as in-vitro model systems, Kidney Int. 48 (1995) 536.

[22] M.J. Wilmer, M.A. Saleem, R. Masereeuw, L. Ni, T.J. van der Velden, F.G. Russel, P.W. Mathieson, L.A. Monnens, L.P. van den Heuvel, E.N. Levtchenko, Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters, Cell Tissue Res. 339 (2010) 449.

[23] A.G. Bodnar, M. Ouellette, M. Frolkis, S.E. Holt, C.P. Chiu, G.B. Morin, C.B. Harley, J.W. Shay, S. Lichtsteiner, W.E. Wright, Extension of life-span by introduction of telomerase into normal human cells, Science 279 (1998) 349.

[24] A.C. Stamps, S.C. Davies, J. Burman, M.J. O'Hare, Analysis of proviral integration in human mammary epithelial cell lines immortalized by retroviral infection with a temperature-sensitive SV40 T-antigen construct, Int. J. Cancer 57 (1994) 865.

[25] G. L'Allemain, S. Paris, J. Pouyssegur, Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na+/H+ antiport, J. Biol. Chem. 259 (1984) 5809.

[26] S. Verkaart, W.J. Koopman, J. Cheek, S.E. van Emst-de Vries, L.W. van den Heuvel, J.A. Smeitink, P.H. Willems, Mitochondrial and cytosolic thiol redox state are not detectably altered in isolated human NADH:ubiquinone oxidoreductase deficiency, Biochim. Biophys. Acta 1772 (2007) 1041.

[27] A. Scharstuhl, H.A. Mutsaers, S.W. Pennings, W.A. Szarek, F.G. Russel, F.A. Wagener, Curcumin-induced fibroblast apoptosis and in vitro wound contraction are regulated by antioxidants and heme oxygenase: implications for scar formation, J. Cell. Mol. Med. 13 (2009) 712.

[28] W.J. Koopman, S. Verkaart, H.J. Visch, F.H. van der Westhuizen, M.P. Murphy, L.W. van den Heuvel, J.A. Smeitink, P.H. Willems, Inhibition of complex I of the electron transport chain causes O2−·-mediated mitochondrial outgrowth, Am. J. Physiol. Cell Physiol. 288 (2005) C1440–C1450.

[29] M. Roest, H. Voorbij, Y. Van der Schouw, P. Peeters, T. Teerlink, P. Scheffer, High levels of urinary F2-isoprostanes predict cardiovascular mortality in postmenopausal women, J. Clin. Lipidol. 2 (2008) 298.

[30] F. Valsecchi, J.J. Esseling, W.J. Koopman, P.H. Willems, Calcium and ATP handling in human NADH:ubiquinone oxidoreductase deficiency, Biochim. Biophys. Acta 1792 (2009) 1130.

[31] Y.S. Yang, R.R. Balcarcel, 96-Well plate assay for sublethal metabolic activity, Assay Drug Dev. Technol. 2 (2004) 353.

[32] F. Santangelo, V. Witko-Sarsat, T. Drueke, B. scamps-Latscha, Restoring glutathione as a therapeutic strategy in chronic kidney disease, Nephrol. Dial. Transplant. 19 (2004) 1951.

[33] S.V. Shah, R. Baliga, M. Rajapurkar, V.A. Fonseca, Oxidants in chronic kidney disease, J. Am. Soc. Nephrol. 18 (2007) 16.

[34] G. Min-Oo, A. Fortin, J.F. Poulin, P. Gros, Cysteamine, the molecule used to treat cystinosis, potentiates the anti-malarial efficacy of artemisinin, Antimicrob. Agents Chemother. (2010).

[35] F. Bellomo, S. Corallini, A. Pastore, A. Palma, C. Laurenzi, F. Emma, A. Taranta, Modulation of CTNS gene expression by intracellular thiols, Free Radic. Biol. Med. (2010).

[36] V. Vitvitsky, M. Witcher, R. Banerjee, J. Thoene, The redox status of cystinotic fibroblasts, Mol. Genet. Metab. (2009).

[37] J.W. Foreman, M.A. Bowring, J. Lee, B. States, S. Segal, Effect of cystine dimethylester on renal solute handling and isolated renal tubule transport in the rat—a new model of the Fanconi syndrome, Metab., Clin. Exp. 36 (1987) 1185.

[38] L.H. Lash, Role of glutathione transport processes in kidney function, Toxicol. Appl. Pharmacol. 204 (2005) 329.

[39] I.M. Frey, I. Rubio-Aliaga, A. Siewert, D. Sailer, A. Drobyshev, J. Beckers, M.H. de Angelis, J. Aubert, H.A. Bar, O. Fiehn, H.M. Eichinger, H. Daniel, Profiling at mRNA, protein, and metabolite levels reveals alterations in renal amino acid handling and glutathione metabolism in kidney tissue of Pept2−/− mice, Physiol. Genomics 28 (2007) 301.

[40] K. Zandi-Nejad, A.A. Eddy, R.J. Glassock, B.M. Brenner, Why is proteinuria an ominous biomarker of progressive kidney disease? Kidney Int. Suppl. 66 (2004) S76–S89.

[41] R. Franco, J.A. Cidlowski, SLCO/OATP-like transport of glutathione in FasL-induced apoptosis: glutathione efflux is coupled to an organic anion exchange and is necessary for the progression of the execution phase of apoptosis, J. Biol. Chem. 281 (2006) 29542.

[42] M. Park, A. Helip-Wooley, J. Thoene, Lysosomal cystine storage augments apoptosis in cultured human fibroblasts and renal tubular epithelial cells, J. Am. Soc. Nephrol. 13 (2002) 2878.

[43] A. Kumar, A.K. Bachhawat, A futile cycle, formed between two ATP-dependant gamma-glutamyl cycle enzymes, gamma-glutamyl cysteine synthetase and 5-oxoprolinase: the cause of cellular ATP depletion in nephrotic cystinosis? J. Biosci. 35 (2010) 21.

[44] S. Miyauchi, E. Gopal, E. Babu, S.R. Srinivas, Y. Kubo, N.S. Umapathy, S.V. Thakkar, V. Ganapathy, P.D. Prasad, Sodium-coupled electrogenic transport of pyroglutamate (5-oxoproline) via SLC5A8, a monocarboxylate transporter, Biochim. Biophys. Acta 1798 (2010) 1164.

[45] V.C. Figueiredo, L.R. Feksa, C.M. Wannmacher, Cysteamine prevents inhibition of adenylate kinase caused by cystine in rat brain cortex, Metab. Brain Dis. 24 (2009) 373.

[46] M.J. Wilmer, J.P. Schoeber, L.P. van den Heuvel, E.N. Levtchenko, Cystinosis: practical tools for diagnosis and treatment, Pediatr. Nephrol. 26 (2011) 205.

[47] M.J. Wilmer, E.I. Christensen, L.P. van den Heuvel, L.A. Monnens, E.N. Levtchenko, Urinary protein excretion pattern and renal expression of megalin and cubilin in nephropathic cystinosis, Am. J. Kidney Dis. 51 (2008) 893.

[48] E.I. Christensen, O. Devuyst, G. Dom, R. Nielsen, S.P. Van der, P. Verroust, M. Leruth, W.B. Guggino, P.J. Courtoy, Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules, Proc. Natl Acad. Sci. USA 100 (2003) 8472.

[49] A. Taranta, M.J. Wilmer, L.P. van den Heuvel, P. Bencivenga, F. Bellomo, E.N. Levtchenko, F. Emma, Analysis of CTNS gene transcripts in nephropathic cystinosis, Pediatr. Nephrol. 25 (2010) 1263.