Brief communication

A futile cycle, formed between two ATP-dependant γ-glutamyl cycle enzymes, γ-glutamyl cysteine synthetase and 5-oxoprolinase: the cause of cellular ATP depletion in nephrotic cystinosis?

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Cystinosis, an inherited disease caused by a defect in the lysosomal cystine transporter (CTNS), is characterized by renal proximal tubular dysfunction. Adenosine triphosphate (ATP) depletion appears to be a key event in the pathophysiology of the disease, even though the manner in which ATP depletion occurs is still a puzzle. We present a model that explains how a futile cycle that is generated between two ATP-utilizing enzymes of the γ-glutamyl cycle leads to ATP depletion. The enzyme γ-glutamyl cysteine synthetase (γ-GCS), in the absence of cysteine, forms 5-oxoproline (instead of the normal substrate, γ-glutamyl cysteine) and the 5-oxoproline is converted into glutamate by the ATP-dependant enzyme, 5-oxoprolinase. Thus, in cysteine-limiting conditions, glutamate is cycled back into glutamate via 5-oxoproline at the cost of two ATP molecules without production of glutathione and is the cause of the decreased levels of glutathione synthesis, as well as the ATP depletion observed in these cells. The model is also compatible with the differences seen in the human patients and the mouse model of cystinosis, where renal failure is not observed.

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1. Introduction

Nephrotic cystinosis is an autosomally determined recessive genetic disorder that results from a defect in the lysosomal efflux of cystine. This is caused by mutations in the gene, *CTNS*, that encodes the transporter cystinosin, which mediates the efflux of cystine from the lysosome to the cytosol. A defect in the *CTNS* gene leads to a high level of cystine accumulation in the lysosome (Town *et al.* 1998). The clinical symptoms are varied and although cystine accumulation is observed in the cells of all organs, it is primarily the kidney that is most severely affected, where a severe renal proximal tubular dysfunction is seen (renal Fanconi syndrome). If untreated in children, it can limit their lifespan to about 10 years. Current therapy includes cystine-depleting therapy (using cysteamine) and renal transplantation (Gahl *et al.* 1987, 2001). However, neither treatment leads to complete recovery. A mouse model has been created by homozygous knockouts of the *CTNS* gene, and these *ctns*$^{-}$/*ctns*$^{-}$ mice showed cystine accumulation in all organs. However, while the mice displayed ocular and other symptoms of the disease, surprisingly, they did not show proximal tubular dysfunction in the kidney or renal failure (Cherqui *et al.* 2002). Using the mouse model, bone marrow transplantations were recently performed and shown to be very successful in the treatment of the disease in mice, raising hopes for a similar treatment being eventually possible in humans (Syres *et al.* 2009).

Several investigations over the past several years have been directed towards understanding how increased lysosomal cystine leads to the clinical symptoms and subsequent manifestations. However, despite many years of study, the pathophysiology of the disease is still not properly

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Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CDME, cystine dimethyl ester; CTNS, cystine transporter; γ-GCS, γ-glutamyl cysteine synthetase; γ-GT, gamma-glutamyl transpeptidase

understood (Baum 1998; Nesterova and Gahl 2008; Wilmer *et al.* 2008).

One suggested mechanism by which intralysosomal cystine accumulation is linked to the clinical manifestations is that the increased cystine levels in the lysosome lead to enhanced apoptosis. This has been based on the 2–4-fold increased rate of apoptosis seen in cystinotic cells, which is reversed when cysteamine treatment decreases the lysosomal cystine levels. It has been suggested that during the early stages of apoptosis when the lysosomal membrane is permeabilized, large amounts of cystine exit the lysosome to enter the cytosol and positively affect the proapoptotic proteins (such as protein kinase Cδ) leading to enhanced apoptosis (Park *et al.* 2002, 2006; Park and Thoene 2005).

A second mechanism that may explain the biochemical basis for the pathophysiology is based on the observation that lysosomal cystine accumulation leads to cellular ATP depletion. ATP depletion in cystinosis was first observed in a cystinosis model (based on loading lysosomes with cystine by cystine dimethyl ester, CDME) (Coor *et al.* 1991). In this study, it was also further suggested that the decreased ATP in cystinotic cells would lead to a decrease in Na+K+-ATPase activity. As this pump generates a Na+ gradient that allows reabsorption of amino acids, phosphate, glucose and other solutes in the proximal tubules of the kidney, defective pumping owing to decreased ATP levels would decrease uptake and thus lead to the severe phenotypes. As the CDME model of cystinosis is not a fully acceptable model owing to the oxidative stress induced by CDME itself, fibroblast cells from cystinotic patients have also been used to investigate the disease. It was observed that significant ATP depletion also occurs in cystinotic fibroblast cells (Levtchenko *et al.* 2006; Wilmer *et al.* 2008). However, whether ATP depletion is a triggering factor leading to the clinical manifestations has not been conclusively established.

2. An integrated explanation to link the intralysosomal accumulation of cystine and the different observations made in nephrotic cystinosis with the pathophysiology is currently missing

The cause behind the decreased ATP levels seen in cystinotic cells was initially thought to be a consequence of a defect in mitochondrial ATP-generating capacity due to the mitochondria possibly becoming dysfunctional in these cells (Baum 1998). However, recent studies have clearly shown that there is no defect in the mitochondrial ATP-generating capacity in cystinotic fibroblasts (Levtchenko *et al.* 2006; Wilmer *et al.* 2008).

So how does ATP depletion occur? Despite ATP depletion being an important and possibly key phenomenon in cystinotic cells, the mechanism by which ATP is depleted in these cells continues to remain a puzzle. Among the possibilities that have been speculated upon include increased ATP consumption or altered glycolysis, but neither of these possibilities have yet been experimentally evaluated (Wilmer *et al.* 2008).

In addition to ATP depletion, cystinotic cells have also been found to have lower levels of glutathione that also might enhance apoptosis in these cells (Laube *et al.* 2006). Furthermore, the levels of cysteine in the cytosol of cystinotic cells are actually lower than normal, despite these cells having high levels of intralysosomal cystine. In the presence of high levels of cystine, measuring the cytosolic cysteine levels has actually been difficult; however, the ability of cysteine precursors to refurbish the low glutathione levels of these cells to normal levels suggests that cytosolic cysteine is indeed limiting in these cells (Chol *et al.* 2004; Mannucci *et al.* 2006). Cysteine is the most limiting nutrient in the glutathione biosynthetic pathway, and it appears that in cystinotic cells they are significantly more limiting (Lu 1999). This is because the lysosomal cystine which is likely to be a pool for cytosolic cysteine is no more available to the cells, thereby leading to cysteine deficiency conditions in the cell despite the high levels of intralysosomal cystine.

Another intriguing observation in patients with nephropathic cystinosis is the almost 60-fold higher levels of 5-oxoproline (pyroglutamic acid) detected in the urine of these patients (Rizzo *et al.* 1999). These high levels of 5-oxoproline were found to return to normal levels when these patients were subjected to cysteamine therapy. The excess 5-oxoproline has been explained as being due to a defect in the γ-glutamyl cycle (Mannucci *et al.* 2006). However, how a defective γ-glutamyl cycle could lead to 5-oxoproline secretion has not been explained. 5-oxoprolinuria (pyroglutamicaciduria) has otherwise been observed in patients who lack the 5-oxoprolinase enzyme or the second enzyme of glutathione biosynthesis (glutathione synthase) (Ristoff and Larsson 2007).

Thus, several aspects of nephrotic cystinosis remain unexplained. Any hypothesis or mechanism that seeks to explain how cystine accumulation in the lysosome leads to the downstream pathogenic events also needs to integrate several different observations: (i) the ATP depletion seen in cystinotic cells although the mitochondrial ATP-generating capacity is intact; (ii) increased levels of apoptosis seen in cystinotic cells; (iii) decreased glutathione levels; (iv) secretion of high levels of 5-oxoproline; and (v) the severe manifestations of the disease specifically seen in the kidney even though cystine accumulates in the lysosomes of other organs to almost similar extents.

3. A futile cycle formed between two ATP-requiring enzymes of the γ-glutamyl cycle as a result of intralysosomal cystine accumulation can explain ATP depletion and other observations made in nephrotic cystinosis

In the present article, we put forward a hypothesis to explain how ATP depletion might occur in cystinotic cells. We propose that ATP depletion results from the formation of a futile cycle involving two ATP-dependant γ-glutamyl cycle enzymes, γ-GCS and 5-oxoprolinase, leading to the continuous depletion of 2 molecules of ATP per cycle. Interestingly, the hypothesis also succeeds in integrating all the other observations seen in cystinosis and is explained below.

The enzyme γ-GCS (also known as glutamate-cysteine ligase) is the first enzyme and the regulated step in glutathione biosynthesis. The reaction involves ligation of glutamate to cysteine and is mediated by ATP leading to the formation of γ-glutamylcysteine (Snoke and Bloch 1954). Investigations into the mechanism of the reaction have revealed that the reaction mechanism involves the formation of activated glutamyl phospate. This intermediate remains in the active site before it attacks the second substrate cysteine leading to the removal of phosphate from the glutamate residue and the formation of γ-glutamyl cysteine, adenosine diphosphate (ADP) and Pi. Interestingly, it has also been shown that the enzyme γ-GCS, if it fails to find the acceptor (cysteine) for γ-glutamyl phosphate, can autocyclize the activated γ-glutamyl phosphate to form 5-oxoproline (pyroglutamate) (Orlowski and Meister 1971).

The sequential events leading to ATP depletion in cystinosis can thus be explained as follows. As a consequence of the defective cystinosin gene, the cell is unable to get sufficient levels of cytosolic cysteine. This can result in the formation of 5-oxoproline through the partial reaction of the γ-GCS enzyme as described above. 5-oxoproline, which is a normal metabolite of the γ-glutamyl cycle, can be acted upon by the action of the 5-oxoprolinase enzyme (another ATP-requiring enzyme) to yield glutamate (Van der Werf *et al.* 1971). Thus, glutamate is cycled by the action of two consecutive ATP-dependant enzymes to yield back glutamate (at the expense of two molecules of ATP). However, 5-oxoprolinase is known to be a very inefficient enzyme (being one of the most sluggish enzymes known with a reaction rate of 0.45 nmol/h) (Watanabe *et al.* 2004) and it is likely that not all the 5-oxoproline generated by this reaction would be converted back into glutamate. Increased levels of 5-oxoproline would thereby accumulate in the cells and the excess would be secreted. This explains the high levels of 5-oxoproline observed in the urine of patients with nephrotic cystinosis (Rizzo *et al.*1999; Mannucci *et al.* 2006). The glutamate that is cycled back can once again feed into the γ-GCS enzyme leading again to the formation of 5-oxoproline in the absence of sufficient amounts of cytosolic cysteine. Thus, by the continuous action of the ATP-dependant γ-GCS and the ATP-dependant 5-oxoprolinase, the cell is forced into a futile cycle leading to ATP depletion. Furthemore, as the cellular glutathione levels are low (owing to lower cytosolic cysteine levels), feedback inhibition of γ-GCS by glutathione would be reduced (Richman and Meister 1975). This would result in greater activity of the enzyme and increase cycling through the futile cycle, thereby leading to continuous ATP depletion. The reduced levels of glutathione in cystinosis could also be a major contributor to the higher rate of apoptosis in cystinotic cells, as proposed earlier (Laube *et al.* 2006).

The highly active γ-glutamyl cycle in the kidney explains the severe renal phenotypes in nephrotic cystinosis. The γ-glutamyl cycle is highly active in the kidney (Meister 1973; Griffith and Meister 1979) since glutathione needs to be made at significant levels for handling the electrophiles encountered, and has also to be effluxed outside the cell (Lash 2005a, b) to allow for amino acid reabsorption through γ-glutamyl transpeptidase. γ-GCS constitutes up to 2–3% of the total kidney protein (Meister 1973). This, and the fact that significant amounts of ATP are needed in these cells for reabsorption of solutes, suggests why the kidney is the most severely affected organ in cystinosis. ATP depletion could lead to malfunctioning of Na+K+-ATPase, leading to problems in reabsorption from the proximal tubules and the consequent effects that account for some of the clinical symptoms seen in nephrotic cystinosis, as proposed earlier (Coor *et al.* 1991). Treatment with cysteamine leads to release of cysteine into the cytosol, and the release of cysteine consequently prevents the channelling of ATP into a futile cycle. This can restore the ATP levels, stop excess 5-oxoproline formation, and also restore glutathione levels, thereby preventing further progression of the disease.

4. The higher γ-glutamyl transpeptidase activity in rodent kidneys (as compared to humans) explains the absence of the severe renal phenotype in ctns^{-}/ctns^{-} mice

Finally, why is it that cystinosis leads to severe renal defects and proximal tubulopathy in humans, but renal failure is not seen in the cystinosis model of mice? A comparison of the enzymes of glutathione degradation in rats, pigs and primates carried out some years ago revealed that in rat, renal activity of membrane-bound γ-glutamyl transpeptidase (γ-GT) (that initiates glutathione degradation) was far higher than in humans, and therefore the rat was not found to be a good model for studying glutathione catabolism in humans (Hinchman and Ballatori 1995). However, importantly, a

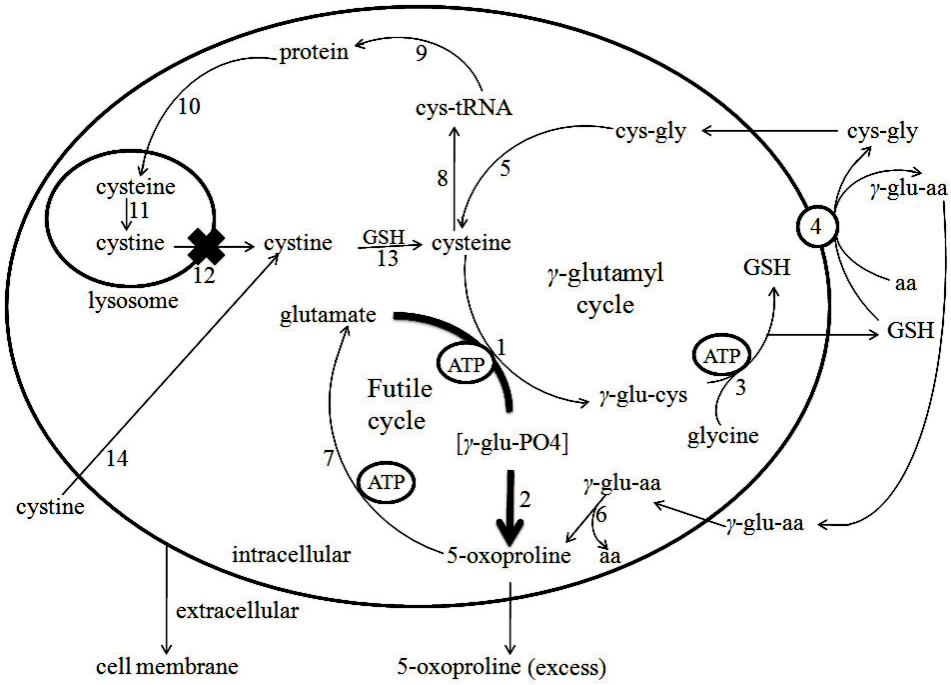

Figure 1. A schematic representation showing how ATP depletion occurs in cystinosis through a futile cycle of two ATP-dependant γ-glutamyl cycle enzymes, γ-glutamylcysteine synthetase and 5-oxoprolinase.

(1) γ-glutamylcysteine synthetase (*EC* 6.3.2.2); (2) Autocyclization of activated glutamate; (3) Glutathione synthetase (*EC* 6.3.2.3); (4) γ-glutamyltranspeptidase (*EC* 2.3.2.2); (5) cinylglycine dipeptidase (*EC* 3.4.3.5); (6) γ-glutamylcyclotransferase (*EC* 2.3.2.4); (7) 5-Oxoprolinase (*EC* 3.5.2.9); (8) Cysteinyl tRNA synthetase (*EC*:6.1.1.16); (9) Protein synthesis; (10) Lysosomal degradation of cellular proteins; (11) Disulphide exchange reaction; (12) Cystine transporter (cystinosin, CTNS); (13) Cystine reductase (*EC* 1.8.1.6); (14) cystine importer. GSH, glutathione; aa, amino acid.

The bold arrow indicates the unusual reaction of γ-glutamylcysteine synthetase under cysteine-limiting conditions that leads to 5-oxoproline formation. **X** indicates a defective cystine transporter seen in cystinosis.

high renal γ-GT in rats would allow extracellular glutathione (from plasma/glomerular filtrates) to be degraded at much higher levels (compared with humans) leading to increased cytosolic cysteine through the action of γ-GT and the cys-gly dipetidases (figure 1). Interorgan glutathione transport is considered to be an important form of cysteine transport (Lash 2005b). The relatively higher cytosolic cysteine levels that would be available in kidney cells (of rat or mice) from this route would prevent the action of the futile cycle from operating even in cystinotic mice, thereby preventing ATP depletion and the consequent severe renal proximal tubulopathy. The futile cycle hypothesis thus also succeeds in explaining the differences seen in human patients with cystinosis and the mouse model of cystinosis.

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References

Baum M 1998 The Fanconi syndrome of cystinosis: insights into the pathophysiology; *Pediatr. Nephrol.* **12** 492–497

Cherqui S, Sevin C, Hamard G, Kalatzis V, Sich M, Pequignot M O, Gogat K, Abitbol M *et al.* 2002 Intralysosomal cystine accumulation in mice lacking cystinosin, the protein defective in cystinosis; *Mol. Cell. Biol.* **22** 7622–7632

Chol M, Nevo N, Cherqui S, Antignac C and Rustin P 2004 Glutathione precursors replenish decreased glutathione pool in cystinotic cell lines; *Biochem. Biophys. Res. Commun.* **324** 231–235

Coor C, Salmon R F, Quigley R, Marver D and Baum M 1991 Role of adenosine triphosphate (ATP) and NaK ATPase in the inhibition of proximal tubule transport with intracellular cystine loading; *J. Clin. Invest.* **87** 955–961

Gahl W A, Reed G F, Thoene J G, Schulman J D, Rizzo W B, Jonas A J, Denman D W, Schlesselman J J *et al.* 1987 Cysteamine therapy for children with nephropathic cystinosis; *N. Engl. J. Med.* **316** 971–977

Gahl W A, Schneider J A and Aula P 2001 A disorder of lysosomal membrane transport; in *The metabolic and molecular basis of inherited disease,* 8th edition (eds) C R Scriver, A L Beaudet, W S Sly and D Valle (New York: McGraw-Hill Press) pp 5085–5108

Griffith O W and Meister A 1979 Glutathione: interorgan translocation, turnover, and metabolism; *Proc. Natl. Acad. Sci. USA* **76** 5606–5610

Hinchman C A and Ballatori N 1990 Glutathione-degrading capacities of liver and kidney in different species; *Biochem. Pharmacol.* **40** 1131–1135

Lash L H 2005a Role of glutathione transport processes in kidney function; *Toxicol. Appl. Pharmacol.* **204** 329–342

Lash LH 2005b Glutathione transport in the kidneys; in *Drug metabolism and transport molecular methods and mechanisms* (ed.) L H Lash (Totowa, New Jersey: Humana Press) pp 319–339

Laube G F, Shah V, Stewart V C, Hargreaves I P, Haq M R, Heales S J and van't Hoff W G 2006 Glutathione depletion and increased apoptosis rate in human cystinotic proximal tubular cells; *Pediatr. Nephrol*. **21** 503–509

Levtchenko E N, Wilmer M J, Janssen A J, Koenderink J B, Visch H J, Willems P H, de Graaf-Hess A, Blom H J *et al.* 2006 Decreased intracellular ATP content and intact mitochondrial energy generating capacity in human *cystinotic* fibroblasts; *Pediatr. Res*. **59** 287–292

Lu S C 1999 Regulation of hepatic glutathione synthesis: current concepts and controversies; *FASEB J*. **13** 1169–1183

Mannucci L, Pastore A, Rizzo C, Piemonte F, Rizzoni G and Emma F 2006 Impaired activity of the γ-glutamyl cycle in nephropathic cystinosis fibroblasts; *Pediatr. Res*. **59** 332–335

Meister A 1973 On the enzymology of amino acid transport; *Science* **180** 33–39

Nesterova G and Gahl W 2008 Nephropathic cystinosis: late complications of a multisystemic disease; *Pediatr. Nephrol.* **23** 863–878

Orlowski M and Meister A 1971 Partial reactions catalyzed by γ-glutamylcysteine synthetase and evidence for an activated glutamate intermediate; *J. Biol. Chem.* **246** 7095–7105

Park M A, Helip Wooley A and Thoene J 2002 Lysosomal cystine storage augments apoptosis in cultured human fibroblasts and renal tubular epithelial cells; *J. Am. Soc. Nephrol.* **13** 2878–2887

Park M A, Peiovic V, Kerisit K G, Junius S and Thoene J G 2006 Increased apoptosis in cystinotic fibroblasts and renal proximal tubule epithelial cells results from cysteinylation of protein kinase C δ; *Am. Soc. Nephrol*. **17** 3167–3175

Park M A and Thoene J G 2005 Potential role of apoptosis in development of the cystinotic phenotype; *Pediatr. Nephrol.* **20** 441–446

Richman P G and Meister A 1975 Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione; *J. Biol. Chem.* **250** 1422–1426

Ristoff E and Larsson A 2007 Inborn errors in the metabolism of glutathione; *Orphanet J. Rare Dis.* **2** 16

Rizzo C, Ribes A, Pastore A, Dionisi-Vici C, Greco M, Rizzoni G and Federici G 1999 Pyroglutamic aciduria and nephropathic cystinosis; *J. Inherit. Metab. Dis.* **22** 224–226

Snoke J E and Bloch K 1954 The biosynthesis of glutathione; in *Glutathione – a symposium* (eds) S Colowic**k**, A Lazarow, E Rac**k**e**r**, D R Schwarz, E Stadtman and H Wa**e**lsch (New York: Acad**e**mic Pr**e**ss) pp 129–141

Syres K, Harrison F, Tadlock M, Jester J V, Simpson J, Roy S, Salomon D R and Cherqui S 2009 Successful treatment of the murine model of cystinosis using bone marrow cell transplantation; *Blood* **114** 2542–2552

Town M, Jean G, Cherqui S, Attard M, Forestier L, Whitmore S A, Callen D F, Gribouval O *et al.* 1998 A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis; *Nat. Genet.* **18** 319–324

Van der Werf P, Orlowski M and Meister A 1971 Enzymatic conversion of 5-oxo-L-proline (L-pyrrolidone carboxylate) to L-glutamate coupled with cleavage of adenosine triphosphate to adenosine diphosphate, a reaction in the gamma-glutamyl cycle; *Proc. Natl. Acad. Sci. USA* **68** 2982–2985

Watanabe T, Abe K, Ishikawa H and Iijima Y l 2004 Bovine 5-oxo-L-prolinase: simple assay method, purification, cDNA cloning, and detection of mRNA in the coronary artery; *Biol. Pharm. Bull*. **27** 288–294

Wilmer M J, van den Heuvel L P, Rodenburg R J, Vogel R O, Nijtmans L G, Monnens L A and Levtchenko E N 2008 Mitochondrial complex V expression and activity in cystinotic fibroblasts; *Pediatr. Res*. **64** 495–497

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