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Ca^{2+} signalling in human proximal tubular epithelial cells deficient for cystinosin

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ABSTRACT

Nephropathic cystinosis is an autosomal recessive lysosomal storage disorder caused by loss-of-function mutations in the *CTNS* gene coding for the lysosomal cystine transporter, cystinosin. Recent studies have demonstrated that, apart from cystine accumulation in the lysosomes, cystinosin-deficient cells, especially renal proximal tubular epithelial cells are characterized by abnormal vesicle trafficking and endocytosis, possible lysosomal dysfunction and perturbed intracellular signalling cascades. It is therefore possible that Ca^{2+} signalling is disturbed in cystinosis, as it has been demonstrated for other disorders associated with lysosomal dysfunction, such as Gaucher, Niemann-Pick type C and Alzheimer's diseases. In this study we investigated ATP-induced, IP_3-induced and lysosomal Ca^{2+} release in human proximal tubular epithelial cells derived from control and cystinotic patients. No major dysregulation of intracellular Ca^{2+} dynamics was found, although ATP-induced Ca^{2+} release appeared slightly sensitized in cystinotic cells compared to control cells. Hence, these subtle changes in Ca^{2+} signals elicited by agonists may contribute to the pathogenesis of the disease.

1. Introduction

Nephropathic cystinosis is a lysosomal storage disorder caused by mutations in the *CTNS* gene that encodes the lysosomal cystine transporter cystinosin [1]. Cystinosis is characterized by intralysosomal storage of cystine leading to cystine accumulation in all organs and tissues. The first affected organ is the kidney. Cystinotic patients present with generalized proximal tubular dysfunction called renal Fanconi syndrome, resulting in the urinary loss of proteins, amino acids, glucose and other solutes [2]. Glomerular dysfunction is also present starting from an early age and is manifested by intermediate and high molecular weight proteinuria and pathological changes in the glomeruli visible in biopsy material [3].

Current therapy of cystinosis is based on a life-long cysteamine treatment [4]. Cysteamine can enter the lysosome, where it reacts with cystine forming products that can exit into the cytoplasm using alternative transporters. The lysosomal storage of cystine is therefore reduced. Started at an early age, cysteamine therapy prevents extra-renal complications and delays the kidney disease progression [5]. However, it is not effective against the main proximal tubular pathology, the renal Fanconi syndrome and the eventual development of kidney damage. Accordingly, it has been hypothesized that cystine accumulation is not the only mechanism of the disease pathogenesis, and that cystinosin might have other functions apart from cystine transport.

The role of endoplasmic reticulum (ER) stress and of the unfolded protein response (UPR) in cystinosis has been revealed recently [6]. UPR signalling is a complex pathway that regulates cell survival in stress conditions and can be caused by the loss of luminal Ca^{2+} from the ER [7]. Previous studies demonstrated elevated levels of oxidized glutathione (GSSG) in cystinotic cells [8–10]. It has been reported that GSSG can increase the Ca^{2+} release from the ER and from the sarcoplasmic reticulum into the cytosol via ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R) [11,12]. Moreover, GSSG was also shown to affect

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some isoforms of the sarco-/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pumps that are responsible for filling the ER stores with Ca^{2+} [13]. Therefore, it is possible that ER Ca^{2+} signalling is affected in cystinosis and may contribute to the pathological process.

Acidic compartments, such as late endosomes and lysosomes, represent another type of intracellular Ca^{2+} stores. Although the capacity of the acidic stores is lower than that of the ER, they play an important function in the regulation of endosomal functioning and fusion and can trigger Ca^{2+} release from larger stores amplifying the signalling cascade [14,15]. The loading of the acidic Ca^{2+} stores is dependent on the luminal pH, but the exact mechanism of their filling remains to be elucidated. The release of Ca^{2+} from the acidic stores is mediated by several channels expressed on the surface of these organelles, including the two-pore channels (TPC1/2/3) that respond to nicotinic acid adenine dinucleotide phosphate (NAADP) [16]. TPCs were shown to play a role in Ca^{2+}-induced Ca^{2+} release through the recruitment of IP_3Rs and RyRs [17]. To date, it has not been investigated whether lysosomal Ca^{2+} signalling or Ca^{2+} stores are affected in nephropathic cystinosis.

A study of the molecular mechanisms of the pathogenesis revealed an altered morphology and function of endosomal and lysosomal compartments in the murine model PTECs deficient for cystinosin [18]. Lysosomes appeared to be abnormally enlarged and lysosomal degradation of proteins was partly impaired. Early studies have demonstrated that lysosomal acidification was unaffected in cystinotic cells, although more recent research questioned these findings [19]. Interestingly, the impaired lysosomal function was demonstrated to be linked to deficient acidic Ca^{2+} stores rather than to lysosomal pH abnormalities in similar pathologies such as Gaucher, Niemann-Pick type C or presenilin deficiency in familial Alzheimer's disease [20–22].

It is therefore important to investigate the ER and the acidic Ca^{2+} stores in cells deficient for cystinosin, particularly the cells suffering the most and causing the earlier and most characteristic clinico-pathological feature of the disease, the renal Fanconi syndrome. In this work we screened for possible alterations in Ca^{2+} signalling in cultured human proximal tubular epithelial cells derived from healthy donors and cystinotic patients.

2. Materials and methods

2.1. Cellular models

The study of the molecular mechanisms of the pathogenesis of cystinosis requires suitable *in vitro* models. To study possible abnormalities in Ca^{2+} signalling, we used cultured conditionally immortalized proximal tubular epithelial cells (ciPTEC) lines obtained from healthy donors and cystinotic patients. The cell lines have been established from living kidney cells exfoliated into the urine providing therefore a non-invasive method of obtaining kidney cell cultures bearing known mutation of the *CTNS* gene leading to nephropathic cystinosis [23]. The cell lines were immortalized using a temperature-sensitive SV40-TERT viral system and subcloned to obtain lines derived from single cells. We have selected 4 control (PT34.8, PT10.5, PT33.5 and PT14.4) and 4 cystinotic (PT2.1, PT46.2, PT53.3 and PT13.5) ciPTEC lines previously characterized by our group [23]. Two of the cystinotic cell lines were bearing a homozygous 57-kb deletion that affects a large part of the *CTNS* gene and results in a complete absence of cystinosin expression at both the mRNA and protein level. The third cell line was compound heterozygous for the 57-kb deletion and the mutation: c.del18_21GACT (p.T7FfsX7) in exon 3, while the fourth cell line was compound heterozygous for the mutations: c.518-519delCA (p.Y173X) in exon 8 and c.1015G > A (p.G339R) in exon 12 of the *CTNS* gene. All four cell lines were derived from patients with a severe clinical form of the disease.

2.2. Cell culture

The cells were grown in DMEM-HAM's F12 culture medium (Lonza) supplemented with 10% foetal bovine serum (Gibco), 50 IU/ml penicillin and 50 mg/ml streptomycin (Lonza). The medium was additionally supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 pg/ml tri-iodothyronine, 36 ng/ml hydrocortisone and 10 ng/ml EGF (all from Sigma).

2.3. ATP-induced Ca^{2+} release in intact cells

Control and cystinotic ciPTEC lines were grown on flat-bottom 96-well plates to 80% confluency. Before the experiment, the cells were washed once with modified Krebs solution (130 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 12 mM glucose and 11 mM HEPES, pH 7.3). Ca^{2+}-free Krebs solution was made identically, but containing no Ca^{2+} and supplemented with 30 mM EGTA. The cells were then loaded with Fura-2 AM (Sigma) (1.25 μg/ml in modified Krebs solution) for 30 min in the dark at room temperature. The cells were washed once with modified Krebs solution and incubated in 100 μl of the same medium for 30 min in the dark to hydrolyze the Fura-2 AM ester. Afterwards, the cells were incubated in EGTA-containing Ca^{2+}-free Krebs solution for 40 s and stimulated with ATP (0.25, 1, 10 and 100 μM in Ca^{2+}-free Krebs solution) at room temperature. The $[Ca^{2+}]$ in the cytosol was recorded with a FlexStation 3 Multi-Mode Microplate Reader from Molecular Devices using the excitation wavelengths of 340 and 380 nm (5 reads per well). Emission was measured at a wavelength of 510 nm. The 340/380 ratio corresponds to the free $[Ca^{2+}]$ in the cytosol. The presented results are the average of 3–4 independent experiments for each cell line.

2.4. Lysosomal Ca^{2+} release

Lysosomal Ca^{2+} measurements in control and cystinotic ciPTEC lines were performed using the protocol described above. Cells were first treated with 2 μM thapsigargin for 2 min to exclude the contribution of the ER Ca^{2+} stores. Ca^{2+} release from the acidic stores was then induced by adding 200 μM L-glycyl-L-phenylalanine 2-naphthylamide (GPN) in Ca^{2+}-free Krebs solution. The amplitude of the Ca^{2+} peak corresponds to the amount of Ca^{2+} stored in the lysosomal compartments disrupted by the GPN reagent.

2.5. IP_3-induced Ca^{2+} release in permeabilized cells

The measurements were performed as described previously [24]. Control and cystinotic ciPTEC lines were plated in 12-well cell-culture plates and grown to confluency. The plates were placed on a thermostated plate at 30 °C on a shaker. The culture medium was aspirated and the cells were permeabilized by incubation in a solution containing 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA and 20 μg/ml saponin for 10 min. The cells were then loaded for 45 min in 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 and 150 nM free $^{45}Ca^{2+}$ (0.3 MBq/ml). The cells were then washed twice in an efflux medium containing 120 mM KCl, 30 mM imidazole (pH 6.8), 1 mM EGTA and 2 μM thapsigargin. One ml of efflux medium was then added and replaced every 2 min. The indicated concentrations of IP_3 or 10 μM of the Ca^{2+} ionophore A23187 were added for 2 min after 8 min of efflux. Eight minutes later, all $^{45}Ca^{2+}$ remaining in the stores was released by incubation with 1 ml of 2% sodium-dodecyl-sulphate for 30 min The $^{45}Ca^{2+}$ present