Metabolic Studies of Rat Renal Tubule Cells Loaded With Cystine: The Cystine Dimethylester Model of Cystinosis[1]

John W. Foreman,[2] Linda L. Benson, Martha Wellons, Ellis D. Avner, William Sweeney, Iiana Nissim, and Itzhak Nissim

J.W. Foreman, Department of Pediatrics, Duke University Medical Center, Durham, NC

L.L. Benson, M. Wellons, Department of Pediatrics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA

E.D. Avner, W. Sweeney, Department of Pediatrics, University of Washington and Children's Hospital, Seattle, WA

I. Nissim, I. Nissim, Department of Pediatrics, University of Pennsylvania and Children's Hospital of Philadelphia, Philadelphia, PA

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ABSTRACT

The cause of Fanconi syndrome in cystinosis is enigmatic. It has previously been shown that renal tubules could be loaded with cystine by incubating them with cystine dimethylester (CDE), mimicking the biochemical hallmark of cystinosis. Such tubules have impaired transport, decreased whole-cell O_2 consumption, and substrate utilization. In this study, the metabolic disturbances in cystine-loaded renal tubule cells were further characterized. Isolated rat renal tubules were loaded with cystine by incubating them with 2 mM CDE for 10 min. This had no significant effect on total ATPase, Na^+-K^+-ATPase, or the ouabain-insensitive ATPase activity of renal tissue homogenates from these cystine-loaded tubules. Intracellular K was significantly lower in the cystine-loaded tubules (37 ± 2 versus 47 ± 3 nEq/mg; $P < 0.008$). Intracellular ATP was reduced by 39% in the cystine-loaded tubules (23.7 ± 2.4 *versus* 38.1 ± 3.3 nmol/mg of protein; $P < 0.0025$). CDE (2 mM) reduced isolated mitochondrial O_2 consumption with glutamate as the substrate by 66% (4.7 ± 0.7 *versus* 13.9 ± 0.8 nm/min per mg of protein, $P < 0.001$) but had no effect on mitochondrial O_2 consumption with succinate as the substrate. It was speculated that the impaired transport from cystine loading with CDE is secondary to a decrease in energy generation.

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[2] Correspondence to Dr. J.W. Foreman, Division of Nephrology-Pediatrics, Duke University Medical Center, Box 3959, Durham, NC 27710.

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Cystinosis is an enigmatic disease characterized biochemically by the excessive storage of cystine intracellularly and clinically by the Fanconi syndrome (1). The intracellular storage of cystine appears to be due to a defective transport system for cystine efflux from lysosomes (2). It is unclear how the intracellular storage of the amino acid cystine leads to the renal tubular dysfunction.

We have previously shown that normal renal tubule cells can be loaded with cystine by incubating them with cystine dimethylester (3). The dimethylester easily traverses the cell membrane and is hydrolyzed, leaving the free amino acid. We and others have shown that this leads to impaired renal transport by these tubules (3,4). Coor *et al.* (5) have shown that loading renal cells with cystine decreases the levels of ATP and that the transport defect in these cystine-loaded cells could be partially corrected when ATP was added to the incubation media. We have also shown that cystine loading decreases the rate of oxidation of metabolic fuels (6). We and others have also shown that this is associated with decreased consumption of O_2 (6,7). These results suggest that an abnormality in energy generation may underlie the transport defect in cystinosis. To characterize further these metabolic abnormalities, we examined the effect of cystine loading on Na^+-K^+-ATPase activity, intracellular ATP concentrations, intracellular potassium concentrations, and mitochondrial oxidation.

METHODS

Preparation of Renal Tubules

Adult male Sprague-Dawley rats weighing 150 to 200 grams were anesthetized, and the kidneys were perfused with Krebs-Ringer bicarbonate buffer with 1 mg/mL collagenase *in situ*. The kidneys were then removed, and the cortex was cut into thin slices with a Stadie-Riggs microtome. The cortical slices were shaken for 30 min in the same collagenase solution at 37°C with the addition of 1 mM alanine, 5 mM glucose, 5 mM glutamine, and 4 mM lactate. The resulting tubules were washed with ice-cold Krebs-Ringer bicarbonate buffer and filtered through two layers of gauze. The tissue was then divided, and half was incubated in Krebs-Ringer bicarbonate buffer under a 95% O_2/5% CO_2 atmosphere containing the above nutrients plus 2 mM cystine dimethylester for 10 min at 37°C to load the cells with cystine; the other half was incubated in buffer alone. After the incubation, the tubules were washed with fresh buffer without cystine dimethylester and were used to measure Na-K ATPase activity, ATP levels, and intracellular potassium content.

ATPase Activity

V_{max} ATPase activity was determined with optimal substrate concentrations on freeze-thawed tubules by the use of the linked pyruvate kinase–lactate dehydrogenase spectrophotometric method (8). The hydrolysis of ATP is coupled to the oxidation of NADH. ATPase activity is calculated from the rate of change of optical density. Specific Na^+-K^+-ATPase activity was determined as the difference between total ATPase activity and the ouabain-insensitive activity.

ATP Measurement

After incubation, the tubules were immediately lysed with ice-cold 6% perchloric acid/1 mM EDTA. The resulting supernates were then neutralized with KOH. ATP was analyzed in the neutralized extracts by use of the linked hexose kinase–glucose-6-dehydrogenase fluorometric method (9).

Intracellular Potassium Levels

The incubated tubules were separated from the buffer by centrifuging through bromododecane into 275 mM sucrose. The tubules were lysed by boiling for 1 min, and potassium in the resulting fluid was analyzed by atomic absorption spectrophotometry with a Perkin-Elmer model 5000 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) (10,11).

Mitochondrial O_2 Consumption

Isolated renal cortical mitochondria were prepared from adult male Sprague-Dawley rats by differential centrifugation (12) in a buffered (pH 7.0) media containing 225 mM mannitol, 75 mM sucrose, 5 mM MOPS, and 50 μM EDTA (MSE). One-half of the mitochondria preparation was incubated at 37°C for 10 min with 2 mM cystine dimethylester in MSE, and the other half was incubated with MSE alone. The mitochondria were then washed with MSE to remove cystine dimethylester from the media and resuspended in buffer containing 150 mM KCl, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 5 mM KH_2PO_4, 0.5 mM EDTA, 5 mM malate, and either 10 mM glutamate or succinate. They were then placed in a sealed chamber at room temperature, and O_2 measurements were made in State 3 respiration polarographically with a Clark electrode (YSI, Yellow Springs, OH) by the addition of ADP to a final concentration of 0.5 mM.

Protein

Tubule protein was measured by the biuret method as described by Gornall *et al.* (13).

Statistics

All results are expressed as the mean ± SE of at least three separate experiments. A *t* test for unpaired data was used to compare two groups for statistical significance. For multiple group comparisons, analysis of variance was used for significance, and posttest comparisons for significance were done with the Tukey test.

RESULTS

As can be seen in Figure 1, renal tubules loaded with cystine by incubation with cystine dimethylester had significantly less intracellular potassium compared with control tubules incubated in buffer alone (37 ± 2 *versus* 47 ± 3 nEq/mg wet wt; $P < 0.008$). The decreased intracellular potassium was not because of direct inhibition of Na^+-K^+-ATPase, as shown in Table 1. The activity of Na^+-K^+-ATPase in the cystine-loaded tubules was higher than that in controls, although this did not reach statistical significance. In contrast, ATP levels were significantly lower in the cystine-loaded tubules (23.7 ± 2.4 *versus* 38.1 ± 3.3 nmol/mg of protein; $P < 0.0025$), as shown in Figure 2.

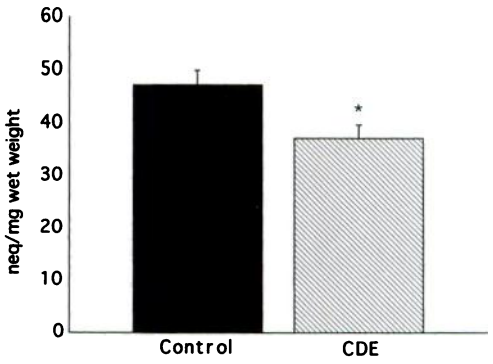

Figure 1. The effect of cystine loading on the intracellular potassium content of renal tubules. Isolated renal tubules were incubated with (hatched bar) and without (solid bar) 2 mM cystine dimethylester (CDE). Each bar represents the mean ± SE of 20 determinations. *$P < 0.008$.

To determine the role of mitochondrial oxidation in the low ATP levels in the cystine-loaded tubules, isolated mitochondria were incubated with different concentrations of cystine dimethylester for 10 min. With glutamate as the substrate, increasing cystine dimethylester concentrations led to a progressive decrease in mitochondrial O_2 consumption (Figure 3). With succinate as the substrate, even 2 mM cystine dimethylester had no significant effect on mitochondrial O_2 consumption (Table 2).

DISCUSSION

We and others have previously shown that normal renal tubules could be loaded with cystine (3,14), the biochemical hallmark of cystinosis, and that these cystine-loaded tubules have decreased O_2 consumption and substrate utilization (2,7). In these studies, we have extended those observations to isolated mito-

TABLE 1. Effect of cystine on ATPase activity[a]

Group	Control	Cystine Loaded
Total ATPase	4.7 ± 1.2	6.2 ± 1.5
Na^+-K^+-ATPase	2.4 ± 0.8	3.8 ± 1.1
Ouabain Insensitive	2.3 ± 0.5	2.4 ± 0.5

[a] Values are picograms of phosphate per minute per milligram of protein. Isolated rat renal cortical tubules were prepared and incubated with and without 2 mM cystine demethylester for 10 min. After incubation, the tubules were assayed for total ATPase, Na^+-K^+-ATPase, and ouabain-insensitive ATPase activity, as described in the Methods section ($N = 7$).

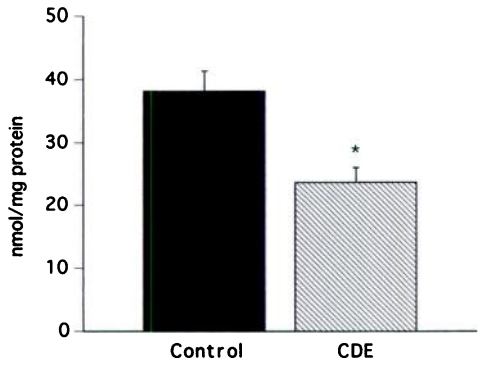

Figure 2. The effect of cystine loading on intracellular ATP levels. Isolated renal tubules were incubated with (hatched bar) and without (solid bar) 2 mM cystine dimethylester (CDE), and the ATP content was measured. Each bar represents the mean ± SE of 10 determinations. *$P < 0.0025$.

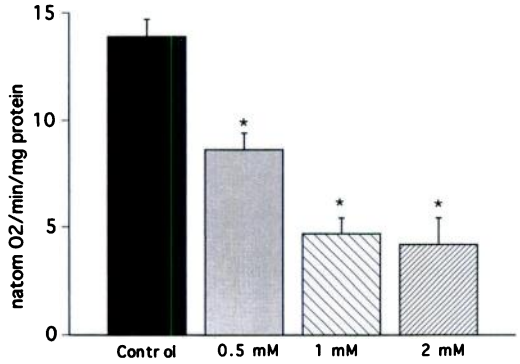

Figure 3. The effect of cystine loading on mitocondrial O_2 consumption with glutamate as the substrate. Isolated mitochondria were incubated with varying concentrations of cystine dimethylester, as stated under each bar, and the consumption of O_2 was measured in State 3 respiration with glutamate as the substrate. Each bar represents the mean ± SE of at least six determinations. *$P < 0.001$ compared with the control value.

chondria. The impairment in mitochondrial oxidation appeared to be at Complex I because there was a decrease in O_2 consumption with glutamate, but not with succinate, which enters the electron transport chain beyond Complex I. Sakarcan *et al.* (7) also found decreased mitochondrial O_2 in intact cells and decreased cellular O_2 consumption directed toward transport. Sakarcan *et al.* (14) also demonstrated that incubating cells with cystine dimethylester led to the swelling of intracellular organelles, consistent with lysosomes, but also led to the swelling of the mitochondria and alterations in their matrix. This physical evidence of mitochondrial changes supports the functional changes in respiration that we and others (7) noted.

We found decreased intracellular levels of potassium after cystine loading and decreased levels of ATP, as have others (5). The activity of Na^+-K^+-ATPase

TABLE 2. Effect of cystine loading on mitochondrial O_2 consumption[a]

CDE Concentration	Glutamate (nm of O_2/min per mg)	Succinate (nm of O_2/min per mg)
Control	13.9 ± 0.8 (N = 18)	6.8 ± 0.8 (N = 5)
0.5 mM	8.6 ± 0.5[b] (N = 18)	
1 mM	4.7 ± 0.7[b] (N = 13)	10.3 ± 2.4 (N = 5)
2 mM	4.2 ± 1.2[b] (N = 6)	5.2 ± 0.9 (N = 5)

[a] Isolated renal cortical mitochondria were prepared and incubated with 2 mM cystine dimethylester (CDE) for 10 min. O_2 consumption was then measured with either 10 mM glutamate or succinate as the substrate, as described in the Methods section.
[b] $P < 0.0001$.

under optimal assay conditions was unaffected by cystine loading, a finding previously reported by Coor *et al.* (5). However, when Na entry into isolated tubules was augmented by the nystatin, a maneuver that increases the activity of Na^+-K^+-ATPase, the expected rise in O_2 consumption was blunted by cystine loading (7). These observations suggest that the activity of Na^+-K^+-ATPase is limited by the supply of ATP; this could affect cellular transport by reducing the electrochemical gradient for Na entry, which is coupled to the entry of many solutes in the proximal tubule. Ben-Nun *et al.* (15) demonstrated directly in LLC-PK_1 cells that cystine loading increased the intracellular concentration of Na and reduced the membrane potential difference. The activity of Na^+-K^+-ATPase in a particulate fraction of the LLC-PK_1 cells was reduced by 50% by cystine loading, in contrast to our results and those of Coor *et al.* (5) in renal tubule cells.

The decreased rates of whole-cell (6,7) and mitochondrial oxidation suggest that the low ATP levels are a consequence of decreased formation rather than increased utilization. Decreased rates of mitochondrial O_2 consumption were observed only with glutamate as the substrate and not with succinate as the substrate. The catabolism of glutamate generates NADH, which enters the respiratory chain at the first enzyme complex (NADH-Q reductase), whereas the catabolism of succinate yields $FADH_2$, which enters at the second enzyme complex (cytochrome reductase). Thus, the reduction in mitochondrial O_2 consumption with cystine loading may be due to the inhibition of the first enzyme complex in the respiratory chain (NADH-Q reductase).

In summary, we found decreased intracellular levels of potassium and ATP in rat renal tubules loaded with cystine by incubating them with cystine dimethylester. We also noted decreased rates of oxidation in mitochondria incubated with cystine dimethylester. We postulate that the decreased solute transport previously noted (3–5) in cystine-loaded tubules is a

result of decreased mitochondrial oxidation and ATP formation, leading to a decrease in the activity of Na^+-K^+-ATPase. This, in turn, reduces the electrochemical gradient for sodium entry. The decreased sodium electrochemical gradient would impair sodium-coupled solute transport. Whether this sequence of events plays a role in the impaired solute transport in cystinosis awaits further investigation, possibly by using renal tubule cells cultured from the urine of patients with cystinosis.

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