Intracellular Distribution of Cystine in Cystine-Loaded Proximal Tubules

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ABSTRACT. Cellular cystine loading with cystine dimethyl ester has been shown to inhibit transport in proximal convoluted tubules perfused *in vitro* and decrease the rate of oxygen consumption in suspensions of proximal tubules. The present study was designed to examine the intracellular distribution of cystine in this model of the Fanconi syndrome of cystinosis and to determine whether cystine or its degradation product, cysteine, is the cytotoxic agent in cystine-loaded rabbit proximal tubules. Tubules were incubated with 2 mmol/L cystine dimethyl ester for 10 min at 37°C and subjected to cellular fractionation. The intralysosomal cystine content (272 ± 125 nmol/mg protein) was significantly higher than that measured in the nucleus (8.7 ± 2.0 nmol/mg protein) and cytosol (9.8 ± 4.0 nmol/mg protein) ($p < 0.05$). Electron micrographs of tubules loaded with cystine depicted large swollen lysosomes. To determine whether cystine or its breakdown product, cysteine, was the cytotoxic agent in tubules incubated with cystine dimethyl ester, the intracellular cystine and cysteine contents were measured and found to be 86.5 ± 14.8 and 5.7 ± 1.7 nmol/mg protein, respectively. These tubules had a 50% decrease in the rate of O_2 consumption. To examine whether the increased level of intracellular cysteine played a role in this decrease in O_2 consumption, we loaded tubules with 2 mmol/L cysteine methyl ester for 10 min. Despite an intracellular cysteine concentration of 59.6 ± 5.8 nmol/mg protein, cysteine-loaded tubules had a rate of O_2 consumption equal to that measured in control tubules. Thus, intracellular cystine loading significantly increases intralysosomal cystine content. The inhibition of tubular respiration with cystine dimethyl ester is due to cystine rather than its degradation product, cysteine. (*Pediatr Res* 35: 447–450, 1994)

Abbreviations

CDME, cystine dimethyl ester
CME, cysteine methyl ester
DTNB, 5,5'-di-thiobis(2-nitrobenzoic acid)

Cystinosis is an autosomal recessive disorder characterized by a high intracellular concentration of cystine (1–3). Cystine accumulates predominantly in lysosomes (2–6), where a defect in the cystine transport has been described (7–10). The accumulation of cystine adversely affects the function of many organs, including the kidney, where cystine accumulation leads to a

generalized abnormality of proximal tubular transport. Although no animal or cell model of cystinosis exists, cells can be loaded with high concentrations of cystine using CDME (11–13). CDME permeates cell membranes where intracellular esterases cleave the methyl esters to liberate high concentrations of cystine.

Previous studies have demonstrated that proximal tubules incubated with CDME increase their cystine concentration to levels comparable to that seen in cystinosis (13). However, the intracellular cystine distribution in proximal tubules incubated with CDME has not been examined. We have previously demonstrated in isolated perfused proximal tubules that addition of CDME inhibited volume absorption, glucose transport, and bicarbonate transport (14). The defect in proximal tubular transport is in part mediated by alterations in proximal tubular metabolism (15, 16). Addition of the methyl esters of tryptophan and leucine did not affect transport, demonstrating that the liberated methanol and amino acid methyl esters did not have a nonspecific toxic effect (14). We also examined the effect of CME on proximal tubule transport and found that although 0.5 mM CME had no effect on proximal convoluted tubule transport, 1.0 and 2.0 mM CME resulted in a significant decrease in volume absorption, bicarbonate, and glucose transport. Thus, high doses of CME produced proximal tubule cytotoxicity *in vitro*. In addition, in the *in vivo* model of Fanconi syndrome produced by intraperitoneal injection of CDME, cysteine was found to be elevated in proximal tubular cells whereas cystine was not detected (13). The purpose of the present study was to examine the cellular distribution of cystine in proximal tubules incubated with CDME. In addition, this study examined whether cystine or its degradation to cysteine was responsible for the inhibition in proximal tubule transport in proximal tubules incubated with CDME.

MATERIALS AND METHODS

Proximal tubule isolation. Rabbit proximal tubules were prepared using the same protocol previously used by our laboratory (16) according to the method described by Weinberg and Humes (17) and Weinberg *et al.* (18). Briefly, adult New Zealand White rabbits were killed, and their kidneys were removed and placed in an ultrafiltrate-like solution containing (in mmol/L) 115 NaCl, 25 NaHCO₃, 2.1 KCl, 2.4 K₂HPO₄, 1.2 MgCl₂, 1.2 MgSO₄, 1.2 CaCl₂. This solution and all solutions were gassed with 95% O₂/5% CO₂ and had a pH of 7.4.

The renal cortex was minced with a razor blade and placed in a silicone-coated flask containing 24 mL of the above ultrafiltrate-like solution except that it contained (in mmol/L) 2.4 CaCl₂, 1.2 KH₂PO₄, 25 mannitol, 5 glucose, 4 Na lactate, 1 alanine, 1 Na butyrate, and 2.5 g/L albumin (Sigma, St. Louis, MO; Fraction V) and 1 g/L collagenase (Boehringer, Mannheim, Indianapolis, IN). The solution was incubated in a shaker bath at 37°C for 35 min. The reaction was arrested by the addition of 75 mL of the above cold ultrafiltrate-like solution (4°C), and the solution was filtered through four layers of gauze. The pellet was

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then washed and resuspended in a 50% Percoll solution (Pharmacia, Piscataway, NJ) containing (in mmol/L) 100 NaCl, 26 NaHCO₃, 3.4 KCl, 1.2 K₂HPO₄, 1.2 MgSO₄, and 2.6 CaCl₂ and centrifuged at 20 400 × *g* for 30 min at 4°C to separate the proximal tubules from glomeruli and distal nephron segments. The proximal tubules were washed and resuspended in a solution (at 5–6 mg protein/mL) containing (in mmol/L) 100 NaCl, 25 NaHCO₃, 2.1 KCl, 2.4 K₂H₂PO₄, 1.2 MgCl₂, 1.2 MgSO₄, 1.2 CaCl₂, 4 Na lactate, 10 Na butyrate, 1 alanine, 5 glucose, and 0.6% dextran. Protein concentrations were measured using the method described by Lowry *et al.* with BSA as the standard. The final proximal tubule preparation contained more than 90% proximal tubules.

Proximal tubule respiration. Proximal tubules were kept at 4°C until use, at which time tubules were warmed to 37°C and bubbled with 95% O₂ and 5% CO₂ for 10 min. To examine the effect of cystine or cysteine loading on proximal tubule respiration, we incubated tubules for 10 min with either 2 mmol/L CDME or CME, respectively. The rate of O₂ consumption was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). After basal O₂ consumption was measured, ouabain (10⁻⁴ mmol/L) was added to inhibit the NaK ATPase to determine the fraction of O₂ consumption rate not used for active transport.

Measurement of intracellular cystine and cysteine. Intracellular cystine and cysteine were determined with the same methods developed by Foreman *et al.* (13). After incubation of tubules in control solutions or in solutions containing either 2 mmol/L CDME or 2 mmol/L CME for 10 min, 5–10 mg of tubules were washed and resuspended in a trichloroacetic acid, EDTA solution, and then homogenized with a Thomas homogenizer (Wheaton, Millville, NJ). The samples were then centrifuged at 1300 × *g* for 10 min at 4°C, and 50 μL of supernatant was added to 150 μL of H₂O, 400 μL of Tris (pH 8.9), and 10 μL of DTNB to form the DTNB derivatives of free thiol groups. Thus, DTNB does not react with cystine. We mixed 50 μL of supernatant with 400 μL of Tris buffer with 0.1 mmol/L DTT to reduce cystine to cysteine. The solution was mixed for 1 h at room temperature, and 10 μL of 10 mmol/L DTNB was added to make the DTNB derivative. Samples were filtered through a 0.22-μm filter, and 20 μL was injected into a Beckman Model 334 HPLC (Beckman, Houston, TX) equipped with a 5-μm Microsorb C₁₈ 4.6 mm × 25 cm column (Rainin Instrument Co., Waburn, MA). A total of 820 mL HPLC grade potassium phosphate (0.2 M), pH 3.0, and 180 mL HPLC grade methanol formed the mobile phase at 0.9 mL/min. The retention time for cysteine-DTNB was 7.5 min. The difference between the pre- and post-DTT reduction assays was due to the cysteine-DTNB derived from cystine. This amount was divided by two to give the concentration of cystine.

Intracellular distribution of cystine. To examine whether there was preferential compartmentalization of cystine in cystine-loaded proximal tubules, we incubated proximal tubules with vehicle or 2 mmol/L CDME for 10 min at 37°C and isolated organelles as previously described (20). Tubules were then disrupted by sonication with a Branson Sonifier Cell Disrupter 200 equipped with a microtip (Branson Sonic Power Co., Danbury, CT). The nuclear fraction was isolated by centrifugation at 800 × *g* for 10 min at 4°C. The supernatant was then centrifuged at 12 000 × *g* for 10 min (4°C) to isolate a lysosome-rich pellet. Samples from the homogenate, 12 000 × *g* supernatant (cytosol), nuclear fraction, and lysosomal fraction were then assayed for intracellular cystine, arylsulfatase activity (21), a lysosomal enzyme marker (21, 22), and protein.

Tubule structure. Pellets of control and cystine-loaded tubules (10 min CDME at 37°C) were processed for electron microscopy according to the method of Gaudio *et al.* (23), with minor modifications. Pelleted tubules were fixed in Karnovsky's paraformaldehyde-glutaraldehyde fixative at least overnight at 4°C. The tubule suspension (100 μL) was then brought to 40°C and mixed with an equal volume of 5% Agar Noble (Difco, Detroit,

MI), which had also been brought to 40°C and was molten. The mixture was then allowed to cool and solidify, after which it was cut into 2-mm³ blocks. The agar blocks were washed in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and postfixed in 1.33% osmium tetroxide in 0.1% sym-collidine buffer for 1 hr at 4°C, followed by repeated washings in 0.1 M sym-collidine. The blocks were dehydrated, and embedding was in Eponate 12 Resin (Ted Pella Inc., Redding, CA).

Semithin and thin sections were cut on a RMC MT 6000 microtome, and the thin sections were stained with 2% uranyl acetate and Reynold's lead citrate. Examination was conducted on a JEOL 100 SC electron microscope at 60 kV.

All data was expressed as mean ± SEM. Statistical analysis was performed with the unpaired *t* test and analysis of variance.

RESULTS

Intracellular distribution of cystine. In the first series of experiments, the intracellular distribution of cystine was examined after incubation with CDME. The results are shown in Table 1. In these studies arylsulfatase was used as a lysosomal marker. The pellet obtained after 12 000 × *g* centrifugation demonstrated a 15-fold enrichment in arylsulfatase activity consistent with significant lysosomal purification. The intracellular cystine concentration was 51-fold higher in this fraction than in the homogenate, consistent with preferential loading of cystine into lysosomes.

Structure. Control proximal tubules and proximal tubules loaded with cystine were examined with electron microscopy. Control proximal tubule cells were of uniform size and retained opposed lateral membranes (Fig. 1). The apical membrane had microvilli, and the basolateral membrane was marked by few small blunt processes. The mitochondria were abundant and regularly spaced with a dense matrix. Small numbers of cytoplasmic vacuoles were observed.

The cystine-loaded tubule cells (Fig. 2) had profound cellular swelling. Although the luminal microvilli were intact, a poorly demarcated, apical, organelle-free zone of cytoplasm existed. The mitochondria remained abundant but were somewhat swollen with irregularities in outline, and the matrix was of nonuniform electron density. There was marked swelling of the vacuolar apparatus (lysosomal system) with abundant, clear cytoplasmic vesicles, some appearing to contain debris. Crystalline inclusions were not present.

Relative cytotoxicity of cystine versus cysteine. The next series of experiments were performed to examine whether intracellular cystine or its degradation product, cysteine, was responsible for the inhibition in proximal tubule respiration. Proximal tubule respiration was measured on cystine loading with 2 mM CDME. As shown in Table 2, a significant inhibition in basal respiration compared with control tubules was observed. Intracellular cystine and cysteine were then measured in cystine-loaded tubules (Table 3). Although cystine increased from undetectable levels to 86.5 ± 14.8 nmol/mg protein, only 5.7 ± 1.7 nmol/mg protein of cysteine was detected. No CDME was detected. To examine whether this small increase in cysteine was responsible for the inhibition in cellular respiration, we loaded proximal tubules with cysteine using CME (Table 3). Despite a 10-fold higher increase in cysteine than that seen with cystine loading, intracellular cysteine loading had no effect on proximal tubule respiration (Table 2).

DISCUSSION

The present study examined the intracellular distribution of cystine in cystine-loaded proximal tubules and whether cystine or its degradation product, cysteine, was responsible for the inhibition in proximal tubule respiration. The data demonstrate that cystine is preferentially loaded into lysosomes and that morphologic studies are consistent with enlargement of lyso-

Table 1. Intracellular compartmentalization of cystine in cystine-loaded proximal tubules ($n = 5$)

	Homogenate	Nuclear	Supernatant (12 000 $\times$ g)	Pellet (12 000 $\times$ g)
Arylsulfatase activity (U/mg protein)	29.3 $\pm$ 13.3	52.2 $\pm$ 21.8	29.1 $\pm$ 6.0	453.8 $\pm$ 188.0*
Enrichment	1	1.7	0.9	15.4
Cystine (nmol/mg protein)	5.3 $\pm$ 2.0	8.7 $\pm$ 2.0†	9.8 $\pm$ 4.0	272 $\pm$ 125.8*
Enrichment	1	1.6	1.8	51.3

* Different from homogenate, nuclear, and supernatant, $p < 0.05$.† $n = 4$. Fig. 1. Control proximal tubule ($\times 3\,300$) showing apical membrane microvilli and uniform cells without swelling. Mitochondria have dense matrix (insert $\times 16\,000$). Fig. 2. Cystine-loaded proximal tubule ($\times 3\,300$) with cell swelling. The apical microvilli are intact, but the mitochondria have an irregular nonuniform matrix (insert $\times 16\,000$). Marked swelling of vacuolar apparatus exists.

Table 2. Proximal tubular respiration in control, cystine, and cysteine-loaded tubules*

	Basal	Ouabain
Control	30.2 $\pm$ 4.8 ($n = 12$)	13.2 $\pm$ 1.3† ($n = 8$)
2 mmol/L CDME	16.4 $\pm$ 1.5‡ ($n = 14$)	12.9 $\pm$ 1.5 ($n = 10$)
2 mmol/L CME	30.8 $\pm$ 3.0 ($n = 9$)	13.1 $\pm$ 1.3† ($n = 9$)

* Values in nmol $O_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.† Different from basal of the same group, $p < 0.001$.‡ Different from basal of control and CME group, $p < 0.01$.

Table 3. Intracellular concentration of cystine and cysteine on incubation with methyl esters*

	Cystine	Cysteine
Control	ND	ND
2 mM CDME	86.5 $\pm$ 14.8	5.7 $\pm$ 1.7
2 mM CME	ND	59.6 $\pm$ 5.8†

* Values in nmol/mg protein. ND, not detected.

† Different from CDME, $p < 0.001$.

somes on cystine loading. This study also demonstrates that cystine rather than its degradation product, cysteine, is responsible for the reduction in cellular respiration in cystine-loaded tubules.

Several studies have demonstrated that cystine accumulates predominantly in lysosomes in cystinosis (2–6). On loading cells with CDME, intracellular esterases cleave the methyl esters to form cystine. The intracellular distribution of cystine after incubation with CDME will be dependent on the distribution of intracellular esterases that cleave the methyl esters to form cystine, which is relatively impermeable. Previous studies have examined the intracellular distribution of cystine in other cells on incubation with CDME. In leukocytes incubated with CDME, Steinherz *et al.* (8) found a 6.7-fold higher cystine concentration in lysosomes compared with the supernatant fraction. Ben-Nun *et al.* (20) found that LLC-PK₁ cells loaded with cystine had a 6.7-fold higher concentration of cystine in the lysosomal pellet than the homogenate. In the present study, where proximal tubules were incubated with 2 mM CDME for 10 min, there was a 51-fold higher concentration in lysosomes compared with the homogenate and a 28-fold higher lysosomal cystine concentration compared with the 12 000 $\times$ g supernatant. These results compare well with the distribution of cystine in leukocytes from patients with cystinosis where a similar cystine distribution was found (2).

Foreman *et al.* (13) demonstrated that the Fanconi syndrome developed in rats that received intraperitoneal injections of CDME for 4 d. However, no renal abnormalities were detected on electron microscopy. These kidneys had a small increase in intracellular cysteine, but no cystine was detected. In the present study, where proximal tubules were incubated with CDME *in vitro*, we observed cellular and lysosomal swelling and alterations in mitochondrial structure. The difference between our *in vitro* study and that where CDME was injected *in vivo* may be the result of the difference in concentration of CDME delivered to the renal tubule and the fact that the kidney tubules *in vivo* may have cleared the intracellular cystine by the time of study.

We have previously examined the respiratory dysfunction in cystine-loaded tubules (16). As in the present study, cystine-loaded proximal tubules had a lower basal rate of O_2 consumption than control tubules. This basal rate of O_2 consumption is comprised of the fraction used for active transport and that for other cellular requirements. As in our previous study, only the NaK ATPase inhibitable fraction of O_2 consumption was affected by cystine loading. Similar results have been demonstrated in ischemic proximal tubules (23).

The present study examined whether the increase in cysteine detected after incubation with CDME was responsible for the

inhibition in transport in cystine-loaded tubules, and thus proximal tubules were loaded with CME. Despite a 10-fold greater increase in cysteine than that found with CDME, intracellular cysteine loading had no effect on the rate of proximal tubule O_2 consumption. The difference between these results and our previous *in vitro* microperfusion studies (14) is likely due to the fact that one tubule was incubated in a 1-cm³ incubation chamber while the bath was constantly being changed. The resultant amino acid loading with an amino acid methyl ester may be many fold higher than that in the present tubule suspension studies.

In summary, the present study demonstrated that cystine is predominantly increased in lysosomes in proximal tubules after incubation with CDME. The small increase in cysteine found in this model of the Fanconi syndrome is not responsible for the decrease in proximal tubule respiration.

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