Cystine accumulation and loss in normal, heterozygous, and cystinotic fibroblasts
(nephropathic cystinosis/amino acid storage/lysosomes)

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ABSTRACT Cystinotic fibroblasts contain approximately 100 times more free cystine than do normal control fibroblasts. When cystinotic fibroblasts were placed in the presence of 30 mM cysteine-glutathione mixed disulfide (CSSG) for 24 hr, their cystine content increased about 3-fold. Similar treatment of normal fibroblasts and fibroblasts from patients heterozygous for cystinosis resulted in a 6- to 7-fold increase in cystine content. In all three cell types, the intracellular free cystine is located within lysosomes. When placed in cystine-free medium after 24 hr in CSSG-containing medium, the normal and heterozygous fibroblasts rapidly lost their lysosomal cystine (t₁/₂ = 20 min), but the cystine content of the cystinotic cells remained stable for over 90 min. In contrast to the findings in intact fibroblasts, cystine loss could not be demonstrated from isolated, cystine-loaded lysosomes from any of the three cell types.

Protein degradation is a major source of lysosomal cystine in cultured skin fibroblasts from patients with nephropathic cystinosis (1). The cystine content of these cells, which is almost 100 times higher than that in normal control fibroblasts (2), can be increased further when high concentrations of disulfide-containing proteins are added to the culture medium (3). Studies of nonprotein sources of cystine in culture medium have been limited by the relative insolubility of cystine. The reduced form of this amino acid, cysteine, is much more soluble, but it oxidizes rapidly and has been reported to be toxic to cultured cells (4, 5).

In this study, the mixed disulfide of cysteine and glutathione (CSSG) was investigated as a soluble source of cyst(e)ine (cystine, cysteine, or both) for cultured cells. We found that high concentrations of this compound increased the cystine content of not only cystinotic fibroblasts but normal and heterozygous (for cystinosis) fibroblasts as well. Using this technique, we studied cystine-loaded cells and lysosomes from each cell type.

METHODS

Cell strains were established from 3-mm skin biopsy specimens by standard techniques. The cells were grown in Coon's modified Ham's F12 medium (KC Biological, Lenexa, KS) which was supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA) (6). Cells were grown at 37°C in either 100-mm plastic dishes (Falcon) or glass roller bottles (Bellco) in an atmosphere of 90% air and 10% CO₂. Medium containing CSSG, glutathione or oxidized glutathione, cysteamine, or chloroquine was neutralized with 5 M NaOH when necessary and sterilized with a 0.22-μm-pore-diameter (Millex) Millipore filter.

Cystine determinations were performed by using a cystine-binding protein assay (7). β-N-Acetyl-D-glucosaminidase, a lysosomal marker, was determined fluorometrically (8). Lysosomal latency studies were performed by spectrophotometric measurement of β-hexosaminidase, using a pyridine buffer with and without the presence of 0.2% Triton X-100 (9). Protein concentrations were determined colorimetrically (10).

Chloroquine, cysteamine, L-cystine, EDTA, N-ethylmaleimide, glutathione and oxidized glutathione, glutathione reductase, NADPH, and Triton X-100 were obtained from Sigma. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside and 4-methylumbelliferone were obtained from Koch-Light Laboratories (Buckinghamshire, England). Triethanolamine and 97% formic acid (3% H₂O by HPLC) were obtained from Eastman. H₂O₂ (30%, wt/wt) was purchased from Mallinckrodt, and Dowex 1X8 anion-exchange resin was purchased from Bio-Rad. L-[³⁵S]Cystine (119 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was obtained from Amersham.

Cystine thiosulfonate was prepared according to the method of Emilozzi and Pichat (11) and was used to synthesize the mixed disulfide of cysteine and glutathione. The disulfide was purified with a Dowex 1X8 formate column. L-[³⁵S]Cystine was used in the synthesis of cystine thiosulfonate in order to prepare the radiolabeled disulfide of cysteine and glutathione (C³⁵SSG).

Prior to incubation with 2 mM C³⁵SSG, cell homogenates were prepared in 10 mM potassium phosphate buffer, pH 7.0. At intervals during the incubation, samples were allowed to react with N-ethylmaleimide and then precipitated with sulfosalicylic acid. The acid-soluble fractions were analyzed after separation by high-voltage electrophoresis (13).

Normal fibroblasts and fibroblasts heterozygous for cystinosis were loaded with cystine by placement for 24 hr in culture medium containing 30 mM CSSG. Cystinotic fibroblasts were initially depleted of their cystine by a 1-hr exposure to 2 mM cysteamine in cystine-free, serum-free medium. The medium was then removed and the plates of cells were washed with phosphate-buffered saline. The cystinotic fibroblasts were then loaded with cystine by placement for 30 min in medium containing 30 mM CSSG. At other times, cystinotic fibroblasts were placed for 24 hr in medium with 30 mM CSSG without prior exposure to cysteamine.

Cell fractionation was performed by modification of the method described by Harms et al. (14). Either dishes or roller bottles of cells were rinsed with phosphate-buffered saline (15) at 4°C and then scraped with a rubber policeman. The cells were collected, centrifuged, resuspended in 10 mM triethanolamine/0.25 M sucrose, pH 7.0, and then lysed by repetitive pipetting in 0.5 ml of the same buffer containing 1 mM EDTA. Nuclear,

Abbreviation: CSSG, mixed disulfide of cysteine and glutathione.
RESULTS

In order to study cell viability, cells were exposed to 30 mM solutions of CSSG, oxidized glutathione, or glutathione for 24 hr. Trypan blue studies at the end of this time showed cell viabilities that were greater than 95% for both normal and cystinotic cells. Cell homogenates incubated with C\textsuperscript{35}S-cysteine. The rates of cysteine formation were similar in the cystinotic and normal cell homogenates. Incubation of cystinotic cells in 30 mM oxidized glutathione or glutathione for 24 hr did not result in any significant changes in intracellular cysteine content or cytosolic glutathione content. Cytosolic fractions from normal, heterozygous, and cystinotic cells exposed to 30 mM CSSG for 24 hr showed a 3-fold increase in glutathione content in all cases (P < 0.005).

Cystinotic cells depleted of 98% of their cysteine with exposure to 1 mM cysteamine (17) reaccumulated 76% of their original cysteine in 24 hr. However, in the presence of 30 mM CSSG, cysteine reaccumulation was enhanced 3- to 4-fold in a 24-hr period. This enhancement was not affected by the presence of 50 mM chloroquine, although this amount of chloroquine usually inhibits the reaccumulation of cysteine by about 80%.

After 24 hr in medium with 30 mM CSSG, the cysteine content of normal fibroblasts increased 6.0-fold, that of heterozygous fibroblasts increased 8.9-fold, and that of cystinotic fibroblasts increased 23-fold. A 3-fold purification of the lysosomal enzyme \( \beta \)-D-glucosaminidase was found in the crude granular fractions of these cell types after fractionation. Cystine concentrations in the cellular fractions paralleled the distribution of \( \beta \)-D-glucosaminidase in all three cell types. When the crude granular fractions of cells that had been incubated in CSSG were compared to those of controls, the cystine in these had increased 9.2-fold, 8.3-fold, and 2.6fold for the normal, heterozygous, and cystinotic cell lines, respectively (Table 1).

Normal and heterozygous fibroblasts that were exposed to 30 mM CSSG for 24 hr and then washed and placed in cystine-free medium lost their increased cysteine content by 90 min (Fig. 1). The cysteine content of these cells after 90-min incubation was equal to that of cells not treated with CSSG. The loss of cystine by normal cells and that by heterozygous cells did not differ significantly. Cystinotic cells treated with cysteamine and then exposed for 30 min to 30 mM CSSG had intracellular cysteine concentrations (0.4-0.9 nmol/mg of protein) similar to those found in the loaded normal and heterozygous cells. These cells showed no significant change in cysteine content for 90 min when placed in cystine-free medium. Cystinotic fibroblasts exposed to 30 mM CSSG for 24 hr had very much increased cysteine levels (approximately 20 nmol/mg of protein) but also maintained their cystine content during a 90-min period in cystine-free medium. These cells lost approximately 25% of their cystine content over the course of 24 hr when placed in cystine-free medium. Similar results were obtained by using cystinotic fibroblasts that had not been exposed to either cysteamine or CSSG. Cystine loss by a cell line from an individual with benign cystinosis showed a pattern of loss similar to that seen in fibroblasts from children with nephropathic cystinosis.

Lysosomes were isolated from cystinotic fibroblasts and from

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### Table 1. Effect of CSSG on cystine content of fibroblasts

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Fraction</th>
<th>Cystine, nmol/mg protein</th>
<th>Hexosaminidase, units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Homogenate</td>
<td>0.11 ± 0.06</td>
<td>99 ± 8</td>
</tr>
<tr>
<td></td>
<td>Granular</td>
<td>0.21 ± 0.20</td>
<td>287 ± 28</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>Homogenate</td>
<td>0.12 ± 0.01</td>
<td>134 ± 11</td>
</tr>
<tr>
<td></td>
<td>Granular</td>
<td>0.32 ± 2.7</td>
<td>275 ± 11</td>
</tr>
<tr>
<td>Cystinotic</td>
<td>Homogenate</td>
<td>7.9 ± 22.4</td>
<td>99 ± 9</td>
</tr>
<tr>
<td></td>
<td>Granular</td>
<td>37.8 ± 98.5</td>
<td>394 ± 410</td>
</tr>
</tbody>
</table>

Plates of confluent cells were placed, for 24 hr, in fresh medium containing 30 mM CSSG or in fresh medium alone. Cystinotic cells were not treated with cysteamine prior to cystine loading. The contents of four plates were combined.

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![Fig. 1. Cystine loss from normal, cystinotic, and heterozygous fibroblasts. Dishes of fibroblasts from three control subjects and three individuals heterozygous for cystinosis were incubated for 24 hr in 30 mM CSSG. Fibroblasts from three cystinotic individuals were examined after treatment with cysteamine followed by a 30-min incubation in 30 mM CSSG. Cells were washed with cystine-free, serum-free medium and then incubated in this medium. Dishes of cells were harvested periodically for analysis of cystine content. Bracketed points indicate the means and standard deviations for three cell lines. Unbracketed points are the means for two cell lines. * Cystinotic cells loaded for 30 min (zero time cystine, 0.7 ± 0.3 nmol/mg of protein, mean ± SD); † heterozygous cells (zero time cystine, 0.8 ± 0.2 nmol/mg of protein); and □, normal cells (zero time cystine, 0.5 ± 0.3 nmol/mg of protein). There is no significant difference between the cystine value for the cystinotic lines at 10 min and the value at 90 min (\( P > 0.2 \) by Student's t test).](image-url)
FIG. 2. Cystine content of isolated granular fractions from cystinotic and cystine-loaded normal fibroblasts. Normal fibroblasts, after 24 hr in 30 mM CSSG, and untreated cystinotic fibroblasts were fractionated and the granular fractions were suspended in buffer. Aliquots were taken for latency studies at the times indicated. The remaining granular fractions were collected by centrifugation and the cystine and protein contents of the pellets were determined. C, A, and G represent normal cell lines; •, A, and G represent cystinotic cell lines. Each point is the mean of three values after correction for latency.

normal fibroblasts that had been treated with CSSG to increase their cystine content. Cystine loss could not be demonstrated from the lysosomes of either cell type when the cells were incubated at 37°C for 30 min (Fig. 2). Lysosomal latency was comparable for the normal and cystinotic lysosomes during the incubation period. Latency after 30 min of incubation ranged between 65% and 95% of the preincubation values for both cystinotic and normal lysosomes.

DISCUSSION

Previous studies have demonstrated that lysosomal protein degradation is an important source of the cystine that accumulates in cystinotic fibroblasts (1). If protein degradation were the only source of lysosomal cystine in these cells, then defective protein catabolism might be responsible for the disorder known as cystinosis. An example might be a defective lysosomal protein–thiol reductase system, as suggested by Griffiths and Lloyd (18). We have shown, however, that cystinotic cells can be made to accumulate cystine in the presence of an inhibitor of lysosomal protein degradation, chloroquine. Cystinotic cells presented with a soluble source of cyst(e)ine, CSSG, can accumulate very large amounts of lysosomal cystine, suggesting that protein degradation is not an exclusive source for lysosomal cystine storage. Accumulation of cystine by normal and heterozygous fibroblasts treated with CSSG suggests that a normal lysosomal mechanism for handling cystine has been overwhelmed.

The differences in the loss of cystine from cystinotic cells and normal cells forced to accumulate cystine are particularly interesting. One possible conclusion is that, under normal circumstances, cystine or a metabolite of cystine rapidly exits the lysosome. Cystinotic cells may have lost the ability either to transport cystine out of their lysosomes or to metabolize cystine to a molecule that readily diffuses out of the lysosome. The retention of cystine by cystinotic cells appears to be a property that is independent of the intracellular cystine level, which has ranged between 0.7 and 21 nmol/mg of protein in our experiments. The similar patterns of cystine loss by normal and heterozygous fibroblasts are consistent with the fact that heterozygous fibroblasts cannot be distinguished from normal fibroblasts on the basis of cystine content (2). The cystine content of heterozygous leukocytes, on the other hand, is 4–5 times greater than that of normal leukocytes (2). Steinherz et al. (19) have used cystine dimethyl ester to load human leukocytes with cystine. The patterns of cystine loss by normal and cystinotic cells were similar to those reported here. However, the rate of cystine loss by heterozygous leukocytes was intermediate between that of the normal and cystinotic leukocytes. Heterozygous fibroblasts seem to differ from heterozygous leukocytes not only in cystine content but also in the response to cystine loading.

Examination by Steinherz et al. (20) of isolated leukocyte lysosomes loaded with cystine dimethyl ester yielded results similar to those reported here. In this preliminary work, cystine efflux was very prolonged compared to the efflux of other amino acids and did not differ between normal and cystinotic leukocyte lysosomes. More recent work with isolated lysosomes from cells treated with cystine dimethyl ester has shown decreased efflux of cystine from cystinotic lysosomes as compared to normal lysosomes. This work has been performed in leukocytes (21) and, in our laboratory, in fibroblasts. The reason for the difference between CSSG- and cystine dimethyl ester-loaded lysosomes is not clear at this time. One problem with using cystine dimethyl ester is that methanol is released during its hydrolysis. On the other hand, it is difficult, using CSSG, to load normal fibroblasts with as much cystine as is usually seen in cultured cystinotic fibroblasts. Further studies are required to examine the loss of cystine by normal lysosomes before a definitive statement can be made about a possible transport defect in cystinosis.

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