Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 290-296

UPTAKE OF CYSTINE BY CYSTINE-DEPLETED FIBROBLASTS FROM PATIENTS WITH CYSTINOSIS

B. States, J. Lee, and S. Segal

Division of Biochemical Development and Molecular Diseases
Children's Hospital of Philadelphia, and the Departments of
Pediatrics and Medicine, University of Pennsylvania School
of Medicine, Philadelphia, PA 19104

Received November 25,1980

SUMMARY

[^{35}S]L-cystine uptake was measured in cultured skin fibroblasts from patients with nephropathic cystinosis, pretreated with cysteamine to deplete their cystine pools. The uptake was greater in the cystinotic cells than in normal cells. The data suggest that the enhanced [^{35}S]-cystine uptake observed in cystinotic cells is not a consequence of disulfide exchange with stored cystine and may be related to the underlying abnormality in this enigmatic disorder.

INTRODUCTION

Cystinosis is a recessively inherited metabolic disorder characterized biochemically by high intracellular content of (nonprotein) cystine within lysosomes (1). Cultured skin fibroblasts from children with this enigmatic disease are an excellent model system for studies because they retain their high levels of intracellular cystine even after continuous subculture (2).

In a previous study in which [^{35}S]L-cystine was incubated with cystinotic cells, we reported that the uptake of the amino acid was faster and more extensive than that observed in normal cell lines (3). The presence of entrapped cystine in cystinotic cells raised a problem in interpretation of the data since it was possible that the high uptake of labeled amino acid was secondary to the presence of the sequestered cystine rather than a primary characteristic of cystinotic cells.

To circumvent the problem it appeared necessary to deplete the cells of cystine prior to the assessment of cystine uptake. In the past, a number of

0006-291X/81/010290-07$01.00/0

disulfide reducing agents have been employed. In some instances, these treatments have met with only partial success, i.e., addition of ascorbic acid depleted cells of 50% of their lysosomal accumulation of cystine (4), cells grown in low-cystine medium failed to divide (5) while those grown in cystine-free medium failed to survive (6). Although dithiothreitol completely depleted cells of intracellular cystine, treatment of prolonged periods led to cell death (7). To date, the most successful means to deplete cystine is by incubating cells either in complete media containing cysteamine for 18 hours or in cystine-free media with cysteamine for 1 to 2 hours (8).

In the present studies we have measured the uptake of [^{35}S]-cystine by cystinotic cells in which the cystine pools have been decreased to normal levels by preincubation with cysteamine. The results which indicate that cystine uptake is more rapid in cysteamine treated cystinotic cells than normal cells similarly treated forms the subject of this report.

MATERIALS AND METHODS

L-cystine, cysteine-HCl, N-ethylmaleimide, and cysteamine were purchased from Sigma Chemical Co., St. Louis, MO. [^{35}S]-L-cystine (specific activity 119 mCi/mmol), and [^{14}C]-urea (specific activity 57 mCi/mmol) were obtained from the Radiochemical Centre, Arlington Heights, IL. [^{14}C]-D-mannitol (s.a. 45 mCi/mmol) and Biofluor were purchased from New England Nuclear, Boston, MA. Fetal bovine serum, trypsin (1:250) and glutamine were purchased from Flow Laboratories, Inc., Rockville, MD. Minimum Eagle's Medium with Earle salts was obtained from M.A. Bioproducts, Walkersville, MD. Brockway bottles were purchased from Brockway Glass Co., Inc., Haddonfield, NJ. Corning tissue culture glassware and A.R. grade reagents were purchased from Fisher Scientific Co., Pittsburgh, PA.

Human skin fibroblasts. Five cell lines from normal children and five cell lines from children with nephropathic cystinosis were initiated from biopsies. All lines from affected patients contained elevated cystine levels characteristic of such cells. The biopsies were subcultured and monitored for microbial contaminants as previously described by us (3). All cell lines were checked also for mycoplasma by 1) the fluorescent Hoechst stain method outlined by Flow Laboratories, Inc. and described originally by Chen (9), and 2) by the method of Levine (10).

Experimental Procedure. Cells were seeded and grown on coverslips contained in individual 35 x 10 mm petri dishes (3). Eighteen hours before study, the media were removed from cells grown to confluency (five days after subculture). One-half of the total number of coverslips of each line were incubated with 2 ml of Minimum Eagle's Medium supplemented with 2 mM glutamine and 20% fetal bovine serum, while the rest of the coverslips were incubated with 2 ml of the media supplemented with 1 mM cysteamine. At the time of the

experiment, the medium was removed and each coverslip was washed three times with Delbecco phosphate buffered saline (0.002 M Pi) pH 7.4 (3). The procedure for determining intracellular fluid space and extracellular fluid space was based on the Foster and Pardee method (11) as modified by us (3). Two further changes in the procedure described by us (3) were the use of 1.0 mM [^{14}C]-D-mannitol (2 μCi/ml) to determine extracellular fluid space and protein measurements by the method of Oyama and Eagle (12). Transport studies were conducted and distribution ratios were calculated as previously described (3). Distribution ratio is cpm/ml intracellular fluid space to cpm/ml extracellular fluid space.

In studies to determine pattern of intracellular radioactivity, sufficient numbers of cells could be obtained only by subculturing and growing each cell line to confluency (within 5 days after subculture) in T-150 Corning flasks. The monolayer of cells in each Corning flask was refed eighteen hours before study by refeeding the cells with 25 ml of complete Minimum Eagles Medium without or with 1 mM cysteamine. The experimental protocol was the following: The media were removed and the cells were washed three times with phosphate buffered saline containing 0.1% glucose. Twenty ml of phosphate buffered saline containing 0.1% glucose and 0.08 mM [^{35}S]-L-cystine (s.a. 2.3 mCi/mmole to 25 mCi/mm) were added to each flask. Incubations were at 37° with gentle shaking for the designated periods of time. The incubations were stopped by removing the media and washing the cell monolayer rapidly three times with 10 ml 0.9% NaCl. Each flask was drained and the monolayer was fragmented by scraping into 4 ml of 40 mM N-ethylmaleimide prepared in 0.01 M phosphate buffer pH 7.0. To be sure that the intracellular sulfhydryl groups had reacted with N-ethylmaleimide, the fragmented cell suspension was transferred to 12 ml conical centrifuge tube and mixed 1 min on a Vortex mixer. Protein was precipitated by addition of 0.5 ml of 50% TCA. After two hours in the cold, the precipitable material was pelleted by centrifugation for 10 min. at 23,000xg. The resulting supernatant was transferred to a 25 ml Erlenmeyer flask where it was shelled in an alcohol-dry ice bath prior to lyophilization. Depending on the initial specific activity of labeled cystine, each sample was resuspended in either 0.25 or 0.50 ml of water. Ten microliters of each sample was counted in Biofluor to determine the total radioactivity. Counting efficiency was found to be 90%.

The percentages of total radioactivity appearing in cystine was determined by using Whatman 3 MM paper and high voltage electrophoresis carried out in a Gilson high voltage Electrophorator Model D attached to a Forma Temp. Jr. water circulator bath to maintain the temperature between 10 and 14°. The run was for 2 hours at 4,000 V in 6.8% formic acid pH 1.9. This technique separates cystine from the N-ethylmaleimide derivatives of cysteine and reduced glutathione but does not adequately separate the latter two substances from each other. Known standards of cystine and the N-ethylmaleimide adducts of cysteine and reduced glutathione were spotted onto paper and run simultaneously. The papers were air dried and the areas containing the standards were identified with ninhydrin. To determine the percentage of radioactivity in cystine, the papers were cut into 1 cm strips which were counted by liquid scintillation. The counts in the cystine area were compared to the total on the paper (3). The counting efficiency of ^{35}S on paper strips covered with phosphor was 50%.

Cystine was quantitated in fibroblasts employing a Beckman 119 amino acid analyzer as previously described (3). Cystine content of cysteamine treated cells was the same as control cells, i.e., less than 0.02 μmoles 1/2 cystine per gram protein, the limit of sensitivity of the analyzer.

RESULTS

Figure 1 shows the uptake of [^{35}S]-L-cystine by untreated cells and cells

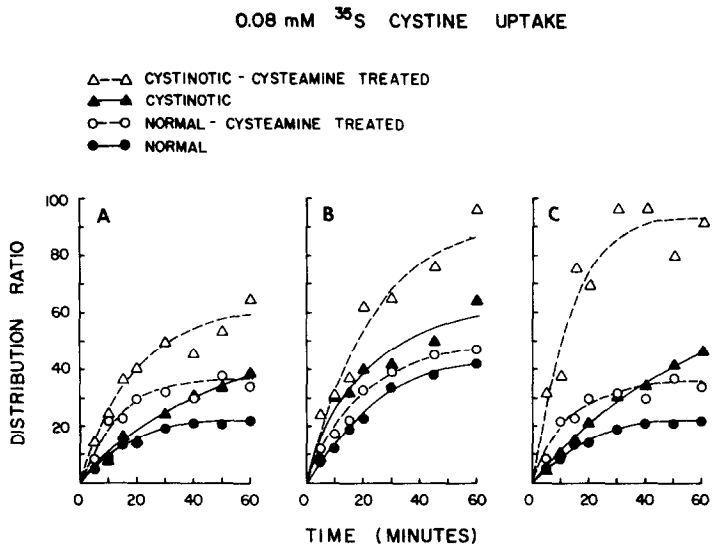

Figure 1. Uptake of 0.08 mM [^{35}S]-L-cystine by cysteamine pretreated and untreated cystinotic and normal cells.
A. Cystinotic A Passage 6
Normal A Passage 8
B. Cystinotic B Passage 16
Normal B Passage 15
C. Cystinotic C Passage 7
Normal C Passage 9
Pretreatment and incubation of coverslips are described under Methods. Distribution ratio is cpm/ml intracellular fluid space to cpm/ml extracellular fluid space.

pretreated with cysteamine. The figure shows, as we have reported previously (3), that untreated cells from cystinotics accumulate label from exogenous [^{35}S]-L-cystine more rapidly and more extensively than untreated cells from normals. The figure shows two important effects of cysteamine pretreatment: 1) cells from cystinotics have increased accumulation of label in comparison with pretreated cells from normals even after depletion of intracellular cystine, and 2) cysteamine pretreated cells from both normals and cystinotics accumulate label more rapidly than their untreated counterparts.

The enhancement of cystine uptake by cysteamine pretreatment appeared to exaggerate the difference between cystinotic and normal cells. This is particularly evident in the experiments shown in B and C in Figure 1. A comparison of distribution ratios at 30 min. in these studies of pretreated normal and cystinotic cells indicates that while normal cells increase an

average 50%, cystinotic cells show an average of 150% difference. Indeed, in two of the three experiments shown (A and C) the initial rate of uptake of cystine by pretreated normal cells was greater than the uptake by untreated cystinotic cells.

The increase of cystine uptake by cysteamine pretreatment is not a general effect on amino acid uptake since we found that glutamate uptake is unaffected while the uptake of proline and lysine by both cystinotic and normal cells is diminished.

We have examined the effects of cysteamine on the pattern of intracellular cystine in the cystinotic cell line shown in experiment B of Figure 1, and a normal cell line over a sixty-min. period of incubation with measurements at 15, 30, 45, and 60 minutes. In the untreated cystinotic cells, the percentage of intracellular radioactivity present in cystine was constant throughout the 60-min. period and approximated 14%. When the cystinotic cells were pretreated with cysteamine, approximately 3% of the label was present as cystine after 15 min. incubation with 0.08 mM [^{35}S]-L-cystine. This 3% level was similar to that found in normal untreated cells throughout 60 min. of incubation. In the pretreated cystinotic cells, the percentage of label in cystine increased such that after 30 min. the percentage was 13%, similar to that found in untreated cystinotic cells. The percentage appearing in cystine, however, continued to increase so that by 60 min., 32% of the intracellular radioactivity was present as labeled cystine. In normal pretreated cells only 7% of label was present as cystine after 60 min.

DISCUSSION

These studies show that the uptake of cystine is more rapid in cells from cystinotics depleted of cystine than in pretreated normal cells. This finding suggests that the more rapid uptake of cystine, which we observed originally in cystinotic as compared with normal cells, is not related directly to the sequestered cystine already present in untreated cystinotic cells. If the

rapid accumulation of label in cystine in cells from cystinotics were primarily the phenomenon of an immediate disulfide exchange with the pre-existing cystine within the lysosome, uptake of exogenous cystine should appear normal in cells from cystinotics depleted of intracellular cystine (13). This is not the case and suggests that the increased uptake of cystine by cystinotic cells may be related to the underlying abnormality responsible for cystine storage.

The explanation for the more avid uptake of cystine by cysteamine pre-treated and normal cells is not known. The effect seems specific for cystine since uptake of other amino acids tested do not show this phenomenon. Our previous report indicated that the V_{max} of cystine uptake (based on 20-min. incubation) was greater in cystinotic cells but the apparent K_t for transport was unchanged (3). We have not yet determined which of these parameters is influenced by cysteamine pretreatment. As shown by us (3) and as reported by Kaye and Nadler (14), although cystinotic cells accumulate more ^{35}S than normal cells after 20-min. incubation with 0.08 mM [^{35}S]-L-cystine, the initial 5-min. uptake of label may not be markedly different between cystinotic and normal lines. However, when cystinotic and normal cells were pretreated with cysteamine, the initial 5-min. uptake of [^{35}S]-L-cystine by pretreated cystinotic cells was significantly above that of pretreated normal cells. The fact that cystinotic fibroblasts have a greater cystine uptake response to cysteamine than normals tends to support the concept that some unusual property of the cystine transport system may exist in cystinotic cells.

The exaggerated cystine uptake response of cystinotic cells pretreated with cysteamine was paralleled by the higher percentage of the ^{35}S present in intracellular cystine. The pretreated cystinotic cells after 60 min. had 32% of label as cystine but pretreated normals had only 7%. Both types of pretreated cells had a greater percentage in cystine than untreated (14% cystinotic vs. 3% normal). It thus seems that the cystinotic cells were re-accumulating their large cystine pool within a relatively short time after the cells were removed from the influence of the reducing agent, cysteamine.

Further study of the distribution of ^{35}S in pretreated normal and cystinotic cell lines to determine the labeling of other intracellular compounds as well as the quantitation of these substances is needed. Our preliminary data on [^{35}S]-cystine reaccumulation by cystinotic cells make it appear unlikely that faulty protein degradation played a major role (15).

ACKNOWLEDGEMENT

This work was supported in part by grant AM 13900 from the National Institutes of Health and by the Whitehall Foundation.

REFERENCES

1. Schneider, J.A., Schulman, J.D. and Seegmiller, J.E. (1978) The Metabolic Basis of Inherited Diseases (J.B.Stanbury, J.B.Wyngaarden and D.S.Fredrickson, eds.) pp. 1660-1682, McGraw-Hill, New York.
2. Schneider, J.A., Rosenbloom, F.M. and Bradley, K.H. (1967) Biochem. Biophys. Res. Comm. 29, 527-531.
3. States, B., Harris, D. and Segal, S. (1974) J. Clin. Invest. 53, 1003-1016.
4. Kroll, W.A. and Schneider, J.A. (1974)Science 186, 1040-1042.
5. Schulman, J.D. and Bradley, K.H. (1971) J. Pediatr. 78, 833-836.
6. Bannai, S., Tsukeda, H. and Okumura, H. (1977) Biochem. Biophys. Res. Comm. 74, 1582-1588.
7. Goldman, H., Scriver, C.R., Aaron, K. and Pinsky, L. (1970) Lancet 1, 811-812.
8. Thoene, J.G., Oshima, R.G., Crawhall, J.C., Olson, D.L. and Schneider, J.A. (1976) J. Clin. Invest. 58, 180-189.
9. Chen, T.R. (1977) Exper. Cell. Res. 104, 255-262.
10. Levine, E.M. (1971) Tissue Culture Manual Procedure 75394, pp. 305-308, Tissue Culture Association, Gaithersburg.
11. Foster, D.O. and Pardee, A.B. (1969) J. Biol. Chem. 244, 2675 -2681.
12. Oyama, V.I. and Eagle, H. (1956) Proc. Soc. Exper. Biol. Med. 91, 305-307.
13. Oshima, R.G., Rhead, W.J., Thoene, J.G. and Schneider, J.A. (1976) J. Biol. Chem. 251, 4287-4293.
14. Kaye, C.I. and Nadler, H.L. (1976) Pediat. Res. 10, 637-641.
15. Thoene, J.G., Oshima, R.G., Ritchie, D.G. and Schneider, J.A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4505-4507.