Biochimica et Biophysica Acta, 367 (1974) 81–87

BBA 76746

ISOLEUCINE TRANSPORT BY CULTURED HUMAN FIBROBLASTS

II. SELECTION OF A CELL LINE WITH REDUCED ISOLEUCINE UPTAKE

RICHARD E. HILLMAN and ELAINE F. OTTO

Department of Pediatrics, Washington University School of Medicine and the Division of Medical Genetics, St. Louis Children's Hospital, St. Louis, Mo. 63110 (U.S.A.)

Received April 5th, 1974)

SUMMARY

Isoleucine uptake was studied in fibroblasts cultured from a patient with a defect in isoleucine metabolism. These fibroblasts after successive passage in a medium low in isoleucine (nutrient Mixture F-12) had been shown previously to lack the sodium-dependent isoleucine uptake seen in normal cell lines. In the present report the cells were shown to undergo additional changes in isoleucine uptake when grown in a medium high in isoleucine (Eagle's minimal essential medium). The fibroblasts were no longer able to concentrate isoleucine. The apparent K_m values for isoleucine were not different from those of the parent F-12 cell line. However, the apparent V values were somewhat reduced and the apparent diffusion constant was over two times greater. The cells, like the parent cell line did not display sodium-dependent uptake and were ouabain insensitive.

INTRODUCTION

There have been a limited number of studies of amino acid uptake in diploid human fibroblasts [1–4]. Three of these studies were directed toward the evaluation of amino acid uptake in fibroblasts grown from patients with known defects in intestinal and/or renal transport of these substances. Recently [4], data was reported from this laboratory indicating that fibroblasts grown from a patient with a defect in isoleucine metabolism [5] accumulated progressively less isoleucine as the cells were passed in culture and that a stable culture finally was obtained which lacked the sodium-dependent isoleucine uptake seen in normal cell lines.

The original biopsy obtained from the patient initially demonstrated virtually complete growth inhibition when cultured in a relatively high isoleucine medium (Eagle's minimal essential medium) and was, therefore, cultured in a low isoleucine medium (nutrient Mixture F-12). However, after several passages in the low isoleucine medium, the resultant culture, which lacked sodium-dependent uptake of isoleucine could be maintained in either nutrient Mixture F-12 or Eagle's medium. The studies reported in this report indicate that a significant change in isoleucine uptake occurred when the cells were transferred to the relatively high isoleucine medium.

METHODS

Fibroblast monolayer cultures were grown to confluency on washed, sterile 11 mm x 22 mm coverslips. Uptake studies utilized methods previously reported [4]. The methods used are quite similar to those previously reported by Platter and Martin [1] and by Foster and Pardee [6]. Incubations were performed by dipping the cells in a reaction mixture consisting of a phosphate buffered balanced salt solution (pH 7.4, 37 °C) containing varying concentrations of L-isoleucine and substrates studied as inhibitors. When substrate concentrations exceeded 10 mM during measurements of diffusion, NaCl was reduced to keep the osmolality of the reaction mixture constant. Sodium-free buffers were prepared by equimolar replacement of NaCl with Tris–HCl, pH 7.4. The isoleucine concentration of the incubation mixture ranged from 0.05–150 mM. All incubations contained 5 μCi of L-[U-^{14}C]isoleucine (New England Nuclear Corp.) per 50 ml incubation mixture.

Cultures of patient's cells were maintained in either nutrient Mixture F-12 (Grand Island Biologicals) or Eagle's minimum essential medium (Grand Island Biologicals). The cultures were uniformly split in a ratio of 1 : 4. Cultures grown in Eagle's medium had passed through 7–10 splits before being studied and have been maintained for more than 12 splits without change in growth rate or signs of senescence.

Cultures of normal cell lines were also grown in nutrient Mixture F-12 or Eagle's medium. In experiments measuring transport in normal cell lines there was no difference found between cells grown in these two media.

RESULTS

Fig. 1 shows the timed uptake by the cells grown from the patient and cultured in Eagle's medium, the original culture from the patient maintained in F-12 medium, and the mean of two normal control cultures. Steady-state conditions were observed between 5 and 10 min. After 10 min incubation, uptake by the two control lines was 4.34 ± 0.23 and 4.05 ± 0.36 nmoles per 100 μg protein. At the same time, uptake by the F-12 cells was 2.65 ± 0.21 nmoles per 100 μg protein and uptake by the cells cultured in Eagle's medium was 1.30 ± 0.05 nmoles per 100 μg protein. Thus, final net uptake by patient's cells grown in Eagle's medium was only about 30 % of the uptake by the control cell lines. The results are expressed as moles per 100 μg cell protein. If the published figures for intracellular water [2] in fibroblasts or those calculated in our laboratory are used, then the ratio of intracellular to extracellular isoleucine cannot be shown to exceed 1 in the cells cultured in Eagles' medium.

Fig. 2 demonstrates uptake by cells cultured in Eagle's medium compared to uptake by the culture maintained in Medium F-12 in the concentration range of 10–150 mM isoleucine used to calculate diffusion. The contribution of mediated uptake of isoleucine in this concentration range is quite small. Using this plot, or the method of Akedo and Christensen [7], the apparent diffusion constant for the cells grown in Eagle's medium is more than two times as great as for the cells maintained in Medium F-12. The apparent diffusion constant differed when the method of Akedo and Christensen was used as compared to reading the values directly from Fig. 2. Using the method of Akedo and Christensen the apparent diffusion constant for the F-12 cells was 0.293 μl/100 μg protein per min and the apparent diffusion constant

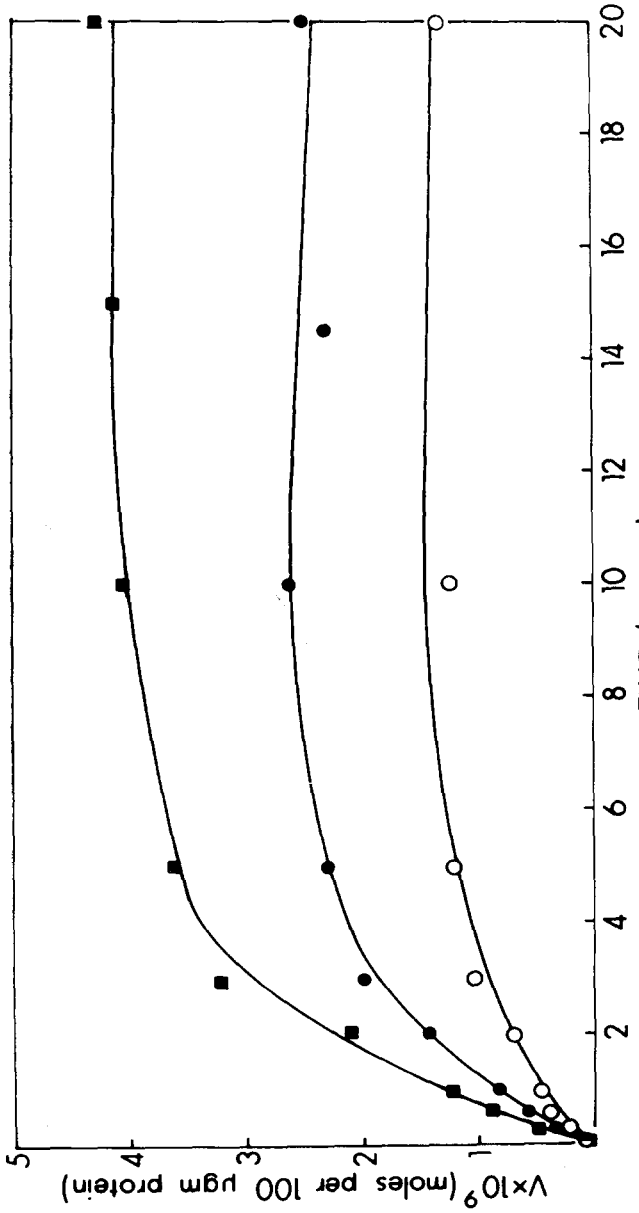

Fig. 1. These plots represent the time course of 0.05 mM isoleucine uptake (V) by fibroblasts grown on coverslips. The upper plot (■–■) represents the mean uptake by two normal cell lines. The center plot (●–●) represents uptake by a cell line grown from a patient with β-ketothiolase deficiency and cultured in nutrient Mixture F-12. The lowest plot (○–○) represents uptake by a cell line derived from the same patient but grown in Eagle's minimal essential medium.

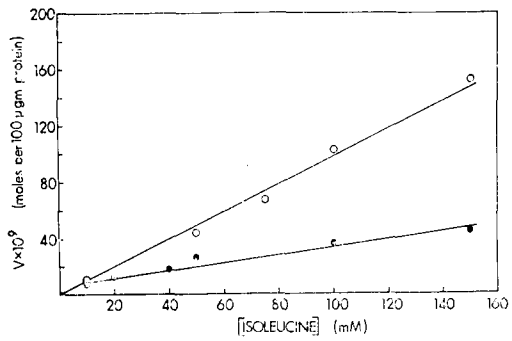

Fig. 2. These plots represent uptake of isoleucine by fibroblasts grown from a patient with a defect in isoleucine metabolism at the high isoleucine concentrations used to calculate an apparent diffusion constant. All incubation mixtures were corrected to keep osmolality constant. The upper curve (○–○) represents uptake by cells cultured in Eagle's minimal essential medium. The lower curve (●–●) represents uptake by cells grown in nutrient Mixture F-12. All incubations were for 1 min.

for the cells grown in Eagle's medium was calculated to be 0.652 μl/100 μg per min. Reading directly from Fig. 2 the values would be 0.325 and 0.945, respectively. The numbers calculated by the method of Akedo and Christensen were used subsequently. Using either method, the diffusion constant in the F-12 cells did not differ from that calculated for the normal control lines.

Fig. 3 demonstrates the uptake of isoleucine by the cells grown in Eagle's medium measured after 1 min and plotted by the double reciprocal method. The solid line represents uptake in an incubation mixture containing a normal amount of sodium. The other points represent uptake by this cell line in a reaction mixture containing a sodium free buffer and in a normal sodium mixture after these cells had been grown to confluency on coverslips and then maintained in F-12 medium for

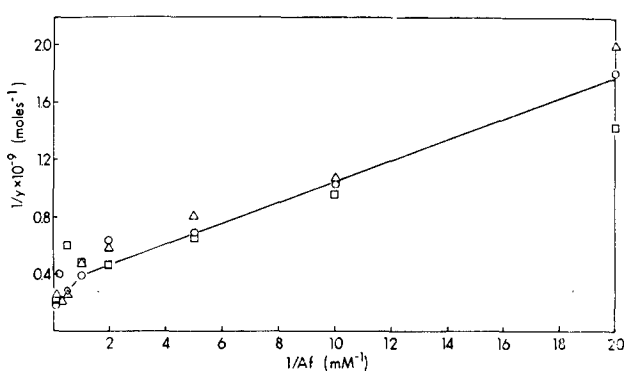

Fig. 3. This plot represents uptake of isoleucine by fibroblasts cultured from a patient with a defect in isoleucine metabolism cultured in Eagle's minimal essential medium plotted by the double reciprocal method. All uptakes were measured after 1 min incubations. The solid line (○–○) represents uptake in a normal sodium-containing phosphate buffer. The other points represent uptake in a sodium-free buffer (Δ) and in normal sodium buffer after the cells had been grown to confluency in Eagle's medium and then incubated in nutrient Mixture F-12 for 24 h before studying uptake (□).

24 h prior to doing the uptake experiments. In contrast to studies performed in normal cell lines [4], no differences in the apparent initial rate kinetics between the cells studied under these three conditions were noted. Thus these cells lacked sodium-dependent uptake of isoleucine. The apparent K_m values for both the low and high concentration limbs of the double reciprocal plot for isoleucine uptake by the cells grown in Eagle's medium are quite similar to those measured in the parent F-12 culture. The apparent V values appeared somewhat lower (Table I). The finding of a second transport system with high values for K_m and V is quite common in mammalian tissues and is unexplained.

TABLE I

APPARENT KINETIC CONSTANTS FOR ISOLEUCINE UPTAKE

The apparent kinetic constants are calculated from double reciprocal plots of uptake measured after 1 min incubations. These data are presented in Fig. 3 for the cells grown in Eagle's medium and were previously published [4] for cells grown in Medium F-12. V expressed as moles/100 μg protein per min (· 10^9).

Growth medium	Low concentration limb*		High concentration limb**	
	K_m (mM)	V	K_m (mM)	V
Nutrient Mixture F-12	0.26	6.8	1.7	14.6
Eagle's minimum essential medium	0.23	3.0	1.3	5.5

* 0.05–1 mM isoleucine.
** 1–10 mM isoleucine.

Efflux of isoleucine was studied after a 10 min incubation in 10 mM isoleucine. The efflux appeared to be considerably greater in the cells grown in Eagle's medium than in the F-12 parent line or the controls. However, even at the zero time point, so little isoleucine was present in the cells grown in Eagle's medium that the studies were not entirely satisfactory.

TABLE II

INFLUENCE OF METABOLIC INHIBITION ON ISOLEUCINE UPTAKE BY FIBROBLASTS GROWN IN EAGLE'S MEDIUM

Confluent fibroblasts grown on coverslips were incubated for 1 min at 37 °C with isoleucine. Ouabain and NaCN were added 10 min prior to the isoleucine.

Experimental conditions	0.1 mM isoleucine		5 mM isoleucine	
	Uptake*	% inhibition**	Uptake*	% inhibition**
Control	0.38	—	3.82	—
Ouabain ($5 \cdot 10^{-4}$ M)	0.50	0	4.00	0
Ouabain (10^{-3} M)	0.48	0	4.77	0
NaCN (10^{-2} M)	0.26	31.6	3.24	15.1

* Uptake $\times 10^9$ (moles per 100 μg protein).
** Percent inhibition as compared to the control uptake.

The effects of ouabain and other metabolic inhibitors were studied at two concentration points (Table II). Like their parent F-12 cells, the cells grown in Eagle's medium were insensitive to ouabain. Similar sensitivity to cyanide was seen in the two cell lines.

Cytogenetic studies were performed on both cell lines using Giemsa and fluorescent banding techniques. The chromosome count remained a normal 46, XX and no deletions or translocations were noted. The cells grown in Eagle's medium could not be distinguished by cytogenetic studies from the parent F-12 cell line.

DISCUSSION

The fibroblasts from the patient with a defect in isoleucine metabolism demonstrate altered isoleucine uptake and also demonstrate that this uptake can be modified by culture conditions. These cells are unable to metabolize isoleucine completely and appear to have undergone adaptation to protect against the toxic effects of this amino acid or its metabolic products. The uptake was previously shown to change as the culture was passed in nutrient Mixture F-12 [4] until a stable culture resulted which lacked sodium-dependent uptake. In the present experiments uptake was altered still more dramatically by subculturing the cells originally grown in Medium F-12 in Eagle's minimal essential medium.

Fibroblasts subcultured in Eagle's medium appeared unable to concentrate isoleucine. The apparent K_m values had not changed from the parent cell line but the apparent V values were somewhat lower and the apparent diffusion constant was over two times as great. The cells seemed to take up isoleucine at a rate similar to the parent cell line but to lose it into the incubation medium rapidly. This change may represent some alteration in the cell membrane which permits increased diffusion of isoleucine. However, because of the difficulty of proving that diffusion is the rate-limiting step [8, 9] an intensification of a low affinity-mediated transport system regulating both influx and efflux may be indicated.

The changes in amino acid uptake seen in these cultures have important implications for other kinds of studies as well as for studies of amino acid uptake. This cell line clearly has responded in some manner to the media in which it was grown, and selection of a growth medium would affect studies of isoleucine catabolism and leucine incorporation into protein.

ACKNOWLEDGEMENTS

This work was supported by U.S.P.H.S. Grant AM 15531 and by the Ranken-Jordan Trust Fund. The authors wish to thank Dr Gurbax Singh Sekhon for the cytogenetic studies and Mrs Sabra Lovejoy for her aid in preparing the manuscript.

REFERENCES

1 Platter, H. and Martin, G. M. (1966) Proc. Soc. Exp. Biol. Med. 123, 140–143
2 Mahoney, M. J. and Rosenberg, L. E. (1970) Biochim. Biophys. Acta 219, 500–502
3 Groth, U. and Rosenberg, L. E. (1972) J. Clin. Invest. 51, 2130–2142
4 Hillman, R. E. and Otto, E. F. (1974) J. Biol. Chem. 249, 3430–3435

5 Hillman, R. E. and Keating, J. P. (1974) Pediatrics 53, 221–225
6 Foster, D. O. and Pardee, A. B. (1969) J. Biol. Chem. 244, 2675–2681
7 Akedo, H. and Christensen, H. N. (1962) J. Biol. Chem. 237, 118–122
8 Inui, Y. and Christensen, H. N. (1966) J. Gen. Physiol. 50, 203–224
9 Wilbrandt, W. and Rosenberg, T. (1961) Pharmacol. Rev. 13, 109–183