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THE INTRALYSOSOMAL pH IN CULTURED HUMAN SKIN FIBROBLASTS IN RELATION TO CYSTINE ACCUMULATION IN PATIENTS WITH CYSTINOSIS

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The intralysosomal pH in cultured skin fibroblasts from a patient with cystinosis and from control fibroblasts was estimated by the method of Hollemans et al. (Hollemans, M., Oude Elferink, R.P.J., de Groot, P.G., Strijland, A. and Tager, J.M. (1981) Biochim. Biophys. Acta 643, 140-151).
1. The intralysosomal pH in cystinotic fibroblasts (5.37 $\pm$ 0.04; n=12) was almost identical to that in the control fibroblasts (5.27 $\pm$ 0.06; n=10).
2. After depletion of cystine by pretreatment of cystinotic fibroblasts with 1 mM cysteamine, there was no significant change in the intralysosomal pH. Incubation of either cystinotic or control fibroblasts with higher concentrations of cysteamine (10 or 25 mM) led to an increase in intralysosomal pH similar to that seen with other weak bases.
3. The fractional volume of the lysosomes in cystinotic fibroblasts (0.100 $\pm$ 0.012; n=4) was higher than that in control fibroblasts (0.039 $\pm$ 0.010; n=4).
4. It is concluded, in contrast to Jonas et al. (Jonas, A.J., Smith, M.L. and Schneider, J.A. (1982) J. Biol. Chem. 257, 13185-13188), that the lysosomal proton-translocating ATPase (Okhuma, S., Moriyama, Y. and Takano, T. (1982) Proc. Natl. Acad. Sci. USA 79, 2758-2762) is not impaired in cystinotic fibroblasts.

Cystinosis is an autosomal recessive disease characterized by intracellular storage of cystine in many body tissues (1). The accumulation of cystine is compartmentalized within the lysosomes (2). Cultured skin fibroblasts from patients with cystinosis have been shown to accumulate cystine (3). The accumulated cystine can be derived either from endogenous (4) or exogenous protein (5) that is degraded in the lysosomes. Intracellular accumulation of cystine in vitro has also been obtained by culturing either control fibroblasts or fibroblasts from patients with cystinosis in the presence of the mixed disulphide of cysteine and glutathione (6) or by incubating control or cystinotic leukocytes with cystine dimethyl ester (7).

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In both systems a rapid efflux of cystine from control cells occurred when they were transferred to medium without the disulphides, whereas there was little, if any, efflux from cystinotic cells.

Very recently (8) Jonas et al. showed that the efflux of cystine from the granular fraction of cystine-loaded normal lymphoblasts is stimulated by ATP in an uncoupler-sensitive manner. These observations led them to suggest that cystine efflux from lysosomes depends on the presence of a proton gradient (8). Jonas et al. (8) also showed that no efflux of cystine occurred from the granular fraction of cystine-loaded cystinotic fibroblasts, whether ATP was absent or present.

We have determined the intralysosomal pH in normal and cystinotic fibroblasts by measuring the distribution of monovalent and divalent weak bases as described earlier (9). Furthermore, we have examined the effect on the intralysosomal pH of β-mercaptoethylamine (cysteamine), a compound that can bring about the depletion of intracellular free cystine in cystinotic fibroblasts (10). The results are described in this paper.

MATERIALS AND METHODS

All experiments were carried out with two fibroblast cell lines kindly provided by Dr J.A. Schneider, San Diego, California. AF2 is a control cell line of foetal origin and FSJ is a cell line obtained from a cystinotic foetus.

Fibroblasts were cultured in Coon's modification of Ham's F12 medium (KC. Biologica Inc., Lenexa, Kansas) supplemented with 10% foetal bovine serum (Boehringer, Mannheim) as described by de Groot et al. (11). For cystine depletion the medium was replaced by freshly-prepared medium containing 1 mM cysteamine (Sigma Chemical Co., St. Louis, Missouri) 1 h prior to harvesting. Both cell lines were harvested by trypsinization at approximately the same stage of confluency. After harvesting, the cells were washed, centrifuged for 5 min at 500 x g, and suspended for at least 0.5 h in Hank's balanced salt solution (HBSS) (Flow Laboratories, Irvine, Scotland) containing 5% dialysed foetal bovine serum. After this treatment the cells were centrifuged again and resuspended in serum-free HBSS.

The free cystine content of the fibroblasts was determined using the cystine-binding protein assay as described in (12).

The distribution of radioactively labelled methylamine, chloroquine and 5,5'-dimethyloxazolidine-2,4-dione (DMO) and sucrose between cells and medium was carried out as described in (9). The harvested fibroblasts (about 1 mg protein/ml) were incubated with 1 ml $^{3}H_2O$ (1.25 μCi/ml) and either [^{14}C]methylamine (0.1 μCi/ml), [^{14}C]chloroquine (0.025 μCi/ml), [^{14}C]DMO (0.1 μCi/ml) or [^{14}C]sucrose (0.2 μCi/ml). The final concentrations of methylamine, chloroquine, DMO and sucrose were 1.8 μM, 0.8 μM, 1.7 μM and 0.32 μM, respectively. The incubation with the labelled compounds was performed at 37 ^{o}C in HBSS containing 1.67 mM $NaHCO_3$, 25 mM 2-(N-morpholino)-

ethane sulphonic acid, 25 mM morpholinopropane sulphonic acid and sufficient Tris to bring the pH to 7.3 or 7.4. The incubation time was 8 min with DMO and sucrose, 12 min with methylamine and 24 min with chloroquine. After incubation, the cells were separated from the medium by a 4-min centrifugation at 12 000 x g and the ^{14}C- and ^{3}H radioactivity in pellet and supernatant were determined as described in (13). Corrections for adhering water were made as described in (14). If necessary, the accumulation factors for chloroquine and DMO were corrected for undissociated base or acid as described by Waddell and Butler (15).

For the theoretical considerations and the method of calculating the fractional volume of the lysosomes and the intralysosomal pH, see (9).

RESULTS

Table 1 shows the distribution of tracer amounts of the labelled weak bases methylamine and chloroquine and the weak acid DMO between the cells and the medium in suspensions of fibroblasts. The accumulation of chloroquine, which is dibasic, was much greater than that of methylamine, which is monobasic. Dibasic bases accumulate to a much greater extent in acid compartments than monobasic bases (see (9,16)). The distribution of DMO is used for calculation of the cytosolic pH (9). From the accumulation of methylamine, chloroquine and DMO one can calculate the volume of the lysosomes as a fraction of the cell volume (P_{lys}) and the intralysosomal pH (pH_{lys}) (see (9)).

The data in Table 1 show that there was a somewhat greater accumulation of chloroquine and methylamine in cystinotic fibroblasts than in control fi-

TABLE I EFFECT OF PRETREATMENT WITH 1 mM CYSTEAMINE ON FREE CYSTINE CONTENT AND INTRALYSOSOMAL pH IN CONTROL FIBROBLASTS AND FIBROBLASTS FROM A PATIENT WITH CYSTINOSIS

Cell line	Addition 1 h before harvesting cells	Cystine content (nmol/mg protein)	Distribution factors			P_{lys}	pH_{lys}
			f_{MA}	f_{CQ}	f_{DMO}		
Control (AF2)	None	< 0.03	5.85,5.64	335,352	1.73,1.97	0.068	5.49
	Cysteamine (1 mM)	0.04	6.56	400,421	1.76,2.71	0.084	5.50
Cystinosis (FSJ)	None	3.4	8.35,8.15	402,430	1.29,1.27	0.114	5.56
	Cysteamine	0.32	10.05,9.38	511,613	1.08,1.24	0.119	5.50

In pretreated fibroblasts, 1 mM cysteamine was added to the culture medium 1 h before harvesting the cells for measurement of intracellular free cystine and the accumulation factor (f) of methylamine (MA), chloroquine (CQ) and 5,5'-dimethyloxazolidine-2,4-dione (DMO). The distribution factors were measured in duplicate at a medium pH of 7.4. Abbreviations: P_{lys}, fractional volume of the lysosomes; pH_{lys}, intralysosomal pH.

broblasts. However, this was not a reflection of a lower intralysosomal pH but of a larger volume of the lysosomes in cystinotic fibroblasts, which has also been reported for granulocytes (7). The calculated intralysosomal pH was almost identical in the two cell lines.

In parallel experiments cysteamine (1 mM) was added to the culture medium 1 h before harvesting the fibroblasts. The third column of Table I shows that this pretreatment caused a 90% loss of free cystine in cystinotic fibroblasts. Before pretreatment, the cystine content of cystinotic fibroblasts was at least 100-fold higher than in control fibroblasts (cf. (3)).

The pretreatment with 1 mM cysteamine had no effect on the intralysosomal pH either in control or in cystinotic fibroblasts (Table I). Since cysteamine is a weak base it might be expected to accumulate in the lysosomes and thereby lead to an increase in intralysosomal pH (16). We therefore determined the effect of adding cysteamine to the incubation medium together with the labelled compounds. Table II shows that the presence in the incubation medium of cysteamine at a concentration of 1 mM had no effect on the intralysosomal pH. However, if the concentration of cysteamine was increased to 10 or 25 mM, there was a very marked decrease in the accumulation of methylamine and chloroquine and hence the calculated intralysosomal pH increased (Table III); the behaviour of cysteamine was thus exactly analogous to that of other monovalent weak bases (9). The extent of the increase in intralysosomal pH was similar in cystinotic and control cells and was not affected by pretreatment of the fibroblasts with 1 mM cysteamine (Table III).

The data of Tables I-III show that the mean fractional volume of the lysosomes in cystinotic fibroblasts is significantly greater than in control fibroblasts (0.100 ± 0.12 (n=4) and 0.039 ± 0.010 (n=4), respectively; $p = < 0.025$). The mean intralysosomal pH under all conditions (except incubation with 10 or 25 mM cysteamine) was 5.37 + 0.04 (n=12) in cystinotic fibroblasts and 5.27 + 0.06 (n=10) in control fibroblasts; this difference is not significant.

Table II EFFECT OF PRESENCE OF 1 mM CYSTEAMINE ON INTRALYSOSOMAL pH IN CONTROL FIBROBLASTS AND FIBROBLASTS FROM A PATIENT WITH CYSTINOSIS

Expt.	Cell line	Addition		Cystine content (nmol/mg protein)	Distribution factors			p_{lys}	pH_{lys}
		1 h before harvesting	during incubation		f_{MA}	f_{CQ}	f_{DMO}		
1.	Control (AF2)	None	None	0.054	4.54	303	1.09	0.037	5.41
		Cysteamine	None	0.096	2.71	119	1.16	0.025	5.52
	Cystinosis (FSJ)	None	None	3.15	8.08	427	1.74	0.112	5.57
		None	Cysteamine		5.74	400	2.58	0.060	5.45
		Cysteamine	None	0.54	4.35	271	1.00	0.035	5.41
		Cysteamine	Cysteamine		4.18	214	1.24	0.045	5.52
2.	AF2	None	None	0.05	3.25	335	1.74	0.020	5.13
		None	Cysteamine		3.43	305	1.99	0.025	5.20
		Cysteamine	None	0.06	3.89	434	1.60	0.022	5.10
		Cysteamine	Cysteamine		4.01	417	1.42	0.023	5.12
	FSJ	None	None	4.3	8.19	797	-	0.065	5.21
		None	Cysteamine		7.12	659	1.91	0.058	5.23
		Cysteamine	None	0.96	9.04	835	1.30	0.075	5.22
		Cysteamine	Cysteamine		8.45	666	1.98	0.082	5.29

Where indicated, 1 mM cysteamine was present in the culture medium 1 h before harvesting the cells or in the incubation medium (pH 7.4 in Expt. 1 and 7.3 in Expt. 2) during measurement for the accumulation factor (f) for chloroquine (CQ), 5,5'-dimethyloxazolidine-2,4-dione (DMO) and methylamine (MA). Abbrevations: p_{lys}, fractional volume of the lysosomes; pH_{lys}, intralysosomal pH.

The fractional volume of the lysosomes in cystinotic fibroblasts appeared to decrease after pretreatment with cysteamine in some experiments (Expt. 1 of Table II and the experiment of Table III) but not in others (Table I and Expt. 2 of Table II). This might depend on culture conditions during pretreatment with cysteamine.

DISCUSSION

Jonas et al. (8) have recentaly shown that the efflux of cystine from cystine-loaded lysosomes isolated from normal lymphoblasts is ATP-dependent and that this efflux does not occur in lysosomes from cystinotic lymphoblasts. They suggest that cystine efflux from lysosomes depends on the functioning

Table III EFFECT OF PRESENCE OF 10 OR 25 mM CYSTEAMINE ON INTRALYSOSOMAL pH IN CONTROL FIBROBLASTS AND FIBROBLASTS FROM A PATIENT WITH CYSTINOSIS

Cell line	Cysteamine		Distribution factors			p_{lys}	pH_{lys}
	in culture medium (mM)	in incubation medium (mM)	f_{MA}	f_{CQ}	f_{DMO}		
Control (AF2)	0	0	5.24	745	1.55	0.029	5.15
	0	10	1.51	38	1.82	0.026	5.72
	0	25	1.05	11	2.02	0.030	5.96
	1	0	6.50	1172	1.50	0.029	5.05
	1	10	1.98	57	1.50	0.032	5.69
	1	25	1.13	14	1.89	0.029	5.91
Cystinosis (FSJ)	0	0	14.40	1843	2.83	0.107	5.23
	0	10	3.44	83	3.50	0.124	5.90
	0	25	1.93	27	4.58	0.113	6.05
	1	0	9.68	1228	2.09	0.070	5.23
	1	10	2.33	68	2.30	0.055	5.77
	1	25	1.27	18	2.61	0.047	5.95

Where indicated, cysteamine was present in the culture medium 1 h before harvesting the cells or in the incubation medium (pH 7.3) during estimation of the accumulation factor (f) for chloroquine (CQ), 5,5'-dimethyloxazolidine-2,4-dione (DMO) and methylamine (MA). Abbreviations: p_{lys}, fractional volume of the lysosomes; pH_{lys}, intralysosomal pH.

of a proton-translocating ATPase. Indeed, Okhuma et al. (16) have produced clear evidence for the presence of an ATP-dependent proton pump in rat-liver lysosomes.

The results reported in this paper show that the intralysosomal pH in cystinotic fibroblasts is the same as that in control fibroblasts. Thus the massive accumulation of cystine in the cystinotic cells is not due to the absence of a proton gradient across the lysosomal membrane.

The results reported by Jonas et al. (8) do not in fact rigorously prove that a proton gradient is involved in the efflux of cystine from lysosomes. Their conclusion is based in part on the observation that carbonyl-cyanide p-trifluoromethoxyphenylhydrazone (CCCP) inhibits cystine efflux from lysosomes in a granular fraction isolated from cystine-loaded normal

lymphoblasts (8). They ascribe the inhibition to the action of CCCP as a protonophore. However, the possibility exists that the inhibition is due to CCCP-induced hydrolysis of ATP by the mitochondria present in the granular fraction and that the cystine-translocating system itself is ATP-dependent. This possibility is supported by their observation that cystine efflux is inhibited by the ATP analogue 5-adenylylimidodiphosphate.

The influence of high concentrations of cysteamine (10 and 25 mM) on the intralysosomal pH agree very well with the influence of other monovalent bases (9). Thus in control fibroblasts the intralysosomal pH is increased from 5.15 to 5.96 by 25 mM cysteamine (Table III) and from 5.28 to 5.93 by 25 mM NH_4Cl (9). From these data it can be concluded that cysteamine behaves like other weak bases and accumulates in the lysosomes in the intact cell. This conclusion is in accordance with the recent demonstration by Kooistra et al. (18) that isolated rat-liver lysosomes are permeable to cysteamine. Indeed, permeation of cysteamine across the lysosomal membrane is required for cystine depletion by cysteamine treatment according to the mechanism proposed by Thoene et al. (10).

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