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Stearylamine permeabilizes the lysosomal membrane to cystine and sialic acid

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Cystine efflux from isolated rat liver lysosomes was enhanced by concentrations of stearylamine that were above the critical micellar concentration. Lysosomal latency, pH, and activity of the proton-translocating ATPase were largely unaffected under controlled experimental conditions. Loss of lysosomal latency was observed at higher stearylamine to protein ratios consistent with a detergent-like mechanism of action. Partially purified cultured fibroblast lysosomes with either defective cystine or sialic acid transport lost their stored material upon exposure to stearylamine. Concentrations of stearylamine which were effective for lysosomal efflux were highly toxic for cultured fibroblasts, thus limiting its use. Under specific conditions, stearylamine apparently selectively permeabilizes the lysosomal membrane. A similar acting, but less toxic agent may be of use in the treatment of lysosomal transport disorders.

Introduction

Lysosomal catabolism of glycoproteins, glycogen and glycosaminoglycans results in the generation of a variety of free sugars and amino acids. These smaller molecular weight substances must then exit the lysosome to reduce osmotic stress and avoid secondary impairment of lysosomal function. Lysosomal transport systems have now been demonstrated for both neutral and cationic amino acids [1–3]. A specific transporter for cystine has also been identified which is uniquely stimulated by divalent cations or polyamines [4–9]. Glucose and other simple sugars appear to share a common system of facilitated diffusion [10–13] while the acetylated sugars, sialic acid (*N*-acetylneuraminic acid) and *N*-acetylglucosamine, are transported by separate mechanisms, respectively [14–16].

Two autosomal recessive human disorders have been attributed to impaired amino acid or carbohydrate lysosomal transport. The first, cystinosis, is a disorder characterized by progressive renal dysfunction resulting from impaired cystine transport [4–7]. The second, sialic acid storage disease, affects neurologic function and is associated with impaired sialic acid transport [14,15]. A clever biochemical approach to the treatment of cystinosis has been devised which is based on the reduction of lysosomal cystine by cysteamine. This reduction results in the formation of a mixed disulfide of cysteamine and cysteine within lysosomes which is then recognized by the functional cationic amino acid transport system [1]. No similar biochemical approach to deplete lysosomes of sialic acid is available. In our studies of the effects of cations on lysosomal cystine transport, we found that stearylamine markedly enhanced lysosomal cystine efflux by a mechanism that appeared to be independent of normal cystine transport. We report our experience using stearylamine to permeabilize the lysosomal membrane.

Methods

Materials. All chemicals were obtained from Sigma Chemical Co. unless otherwise noted. L-[^{35}S]Cystine was obtained from the Amersham Corporation. Tetrahydrofuran and 1,6-diphenyl-1,3,5-hexatriene were obtained from Aldrich Chemical Co. Cystine binding protein was the generous gift of Dr. Jerry A. Schneider. Percoll was obtained from Pharmacia.

Lysosomal purification. Lysosomes were prepared from homogenized rat liver by differential centrifugation and Percoll gradient centrifugation as previously described [8]. Lysosomes were partially purified from cultured fibroblasts by differential centrifugation [17].

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Assays. Latency was determined spectrophotometrically by comparing the ability of lysosomal preparations to hydrolyze *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in the presence (100% broken) and absence (latent preparation) of 0.1% Triton X-100 [23]. Lysosomal pellets from transport studies were collected by centrifugation and routinely assayed for β-hexosaminidase activity using a fluorometric assay [18]. Protein was determined spectrophotometrically [19]. Critical micellar concentration was determined fluorometrically using 5 μM 1,6-diphenyl-1,3,5-hexatriene [20]. Fluorescence was monitored at excitation 358, emission 430, slits 1 and 20 nm. Free-sialic acid was measured fluorometrically following reaction with pyridoxamine [21]. Fluorescence was monitored at excitation 340 nm and emission 410 nm, slits 5 and 5. Cystine was measured using a specific binding protein assay [22].

Cell culture. Fibroblasts were grown in 100 mm plastic dishes using Coons modified Hams F-12 medium supplemented with glutamine and 10% fetal bovine serum. An atmosphere of 10% CO_2, 90% air was employed.

Cystine dimethyl ester. Cystine dimethyl ester was prepared from L-[^{35}S]cystine (Amersham Corp.) by incubation for 24 h at room temperature in methanolic HCl [8]. The product was dried by rotary evaporation and resuspended in methanol. Purity was assessed using thin-layer chromatography (TLC) on cellulose acetate with a methanol/water/formic acid (70:28:2, v/v) solvent [8].

Lysosomal cystine loading. The Percoll gradient fraction containing lysosomes (0.7 ml) was incubated for 10 min at 25°C with 80 μM cystine dimethyl ester (purity > 98%) and then diluted 10-fold with ice-cold buffer followed by centrifugation for 10 min at 10000 × *g*. The lysosomal pellet was washed once and resuspended in 1 ml cold buffer for use in transport studies. Analysis of lysosomes at this point by TLC showed that 98% of the radiolabel was in the form of cystine.

Lysosomal cystine efflux. Lysosomes were incubated in microcentrifuge tubes at 25°C in 1 ml 0.25 M sucrose, 20 mM Hepes, pH 7.0 with 0.2 mg/ml of human serum albumin. Stearylamine was prepared as a 5 mM stock solution in methanol and was added as a 10 μl aliquot. Controls contained 10 μl methanol without stearylamine. After incubation, lysosomes were collected by centrifugation in a microcentrifuge for 2 min. The pellet was broken by sonication in 500 μl of distilled water and assayed for β-hexosaminidase activity and protein content. A unit of β-hexosaminidase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min at 37°C. Aliquots were also taken for cystine assay or scintillation counting where appropriate.

Lysosomal sialic acid efflux. Lysosomes were treated as for measurement of lysosomal cystine efflux except that sonicates were assayed for free-sialic acid content. Values were normalized with respect to β-hexosaminidase activity.

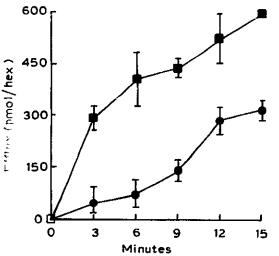

Fig. 1. Efflux of L-[^{35}S]cystine at 25°C from cystine dimethyl ester treated rat liver lysosomes (approx. 100 μg protein) incubated in 0.25 M sucrose, 20 mM Hepes, pH 7.0 (●) and with 50 μM stearylamine (■). Error bars indicate standard deviations which are too small to visualize for one point.

Lysosomal pH. Lysosomal pH was determined fluorometrically using lysosomes obtained from rats which had been intraperitonealy injected with fluoresceinated dextran, $M_r = 70000$, 24 h earlier [15,24]. The relative fluorescence of lysosomes with and without Triton X-100 was compared to the relative fluorescence of fluoresceinated dextran at varying pH.

Results

An initial, rapid loss of cystine from cystine-loaded, Percoll gradient purified, rat liver lysosomes was observed using 50 μM stearylamine (Fig. 1). Concentrations of methanol equivalent to those used to solubilize stearylamine (1%), had no effect on cystine efflux from rat liver lysosomes (24 ± 3 pmol/min per unit β-hexosaminidase vs. 27 ± 1 pmol/min per unit β-hexosaminidase, mean ± S.D.). Incubation of lysosomes with varied concentrations of stearylamine showed that cystine efflux increased only when the stearylamine concentration was greater than 20 μM (Fig. 2). There was no additional effect derived from prolongation of the incubation interval (data not shown). Total β-hexosaminidase activity in pelleted lysosomal material was virtually identical for all samples over the concentration range consistent with stable lysosomal integrity (Fig. 2).

The finding of a concentration threshold effect suggested a detergent-like mechanism of action. Titration with HCl revealed a p*K* of 8.5 indicating that stearylamine is a weak base. Measurement of the critical micellar concentration of stearylamine using 1,6-di-

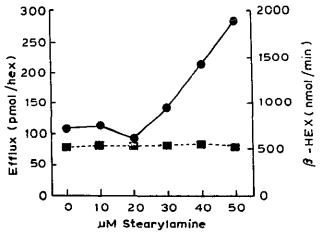

Fig. 2. Efflux of L-[^{35}S]cystine from cystine dimethyl ester treated rat liver lysosomes (approx. 120 μg protein) incubated with increasing stearylamine concentration (●). Incubations were for 10 min as in Fig. 1. Sedimentable β-hexosaminidase activity (■) is indicated by the right sided axis.

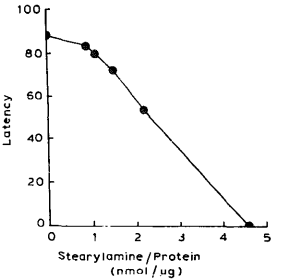

Fig. 4. Latency of rat liver lysosomes incubated for 15 min at 25°C in 0.25 M sucrose, 20 mM Hepes, pH 7.0. The concentration of stearylamine was kept constant at 50 μM while the protein concentration was varied. Latency was determined using *p*-nitrophenyl *N*-acetyl-D-glucosaminide substrate as in Methods.

phenyl-1,3,5-hexatriene yielded a value of approximately 16 μM, roughly correlating with the minimum concentration required for effects on lysosomal cystine content (Fig. 3). It was noted that activity of β-hexosaminidase was markedly decreased in pelleted rat liver lysosomes that had been exposed to 100 μM stearylamine suggesting loss of latency. Latency studies performed with *p*-nitrophenyl substrate showed that lysosomal latency varied directly with the ratio of the concentrations of stearylamine to lysosomal protein (Fig. 4). Lysosomal protein concentrations were therefore determined and adjusted for subsequent experiments to prevent loss of latency.

In order to determine whether stearylamine induced lysosomal proton leakage, the pH of rat liver lysosomes was examined. The pH of FITC-dextran loaded lysosomes was 5.12 ± 0.04 and increased slightly to 5.35 ± 0.05 when 50 μM stearylamine was added. Lysosomes in the presence of stearylamine were acidified by 0.5 pH units upon the addition of 2 mM $MgCl_2$/ATP demonstrating the continued function of the lysosomal proton-translocating ATPase (Fig. 5).

Results with partially purified lysosomes from cultured human fibroblasts were similar to those observed with rat liver lysosomes. The rate of stearylamine-induced cystine loss from cystine-loaded fibroblast lysosomes was far greater than that observed when the cystine transporter was maximally stimulated by 2 mM of spermidine (Table I). Fibroblast lysosomes from an individual with the autosomal recessive disorder

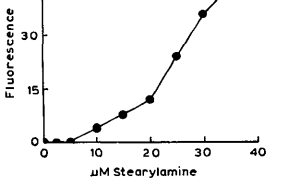

Fig. 3. Determination of the critical micellar concentration of stearylamine using the fluorescence of 5 μM 1,6-diphenyl-1,3,5-hexatriene. Curve extrapolation yields an estimated critical micellar concentration of 16 μM.

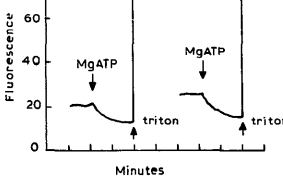

Fig. 5. Acidification by MgATP of fluoresceinated isothiocyanate dextran-loaded, rat liver lysosomes incubated in 0.25 M sucrose, 20 mM Hepes, pH 7.0 alone (A) and with 50 μM stearylamine (B). Each interval represents one minute.

TABLE I

Effects of spermidine and stearylamine on cystine efflux from fibroblast lysosomes

Granular fractions from normal (cystine dimethyl ester treated) and cystinotic fibroblasts were incubated for 15 min at 25°C in 0.25 M sucrose, 20 mM Hepes, pH 7.0 with the indicated additions. Initial values were 1.8 nmol cystine per unit β-hexosaminidase for normal and 75 nmol cystine per unit β-hexosaminidase for cystinotic granular fractions. Values are means ± S.D.

Condition	Normal		Cystinotic	
	pmol/min per unit β-hexosaminidase	content (%)	nmol/min per unit β-hexosaminidase	content (%)
Unincubated	–	100 ± 4	–	100 ± 3
Control	27 ± 4	82 ± 2	0.0 ± 0.0	100 ± 4
2 mM spermidine	45 ± 7	69 ± 5	0.2 ± 0.1	91 ± 3
50 μM stearylamine	134 ± 1	9 ± 1	1.4 ± 0.4	42 ± 15

TABLE II

Effect of stearylamine on the sialic acid content of lysosomes

Crude granular fractions from sialic acid storage disease fibroblasts were incubated for 30 min at 25°C in 0.25 M sucrose, 20 mM Hepes, pH 7.0. Following incubation, samples were centrifuged and the pelleted material was assayed for free-sialic acid content and β-hexosaminidase activity as in Methods. Values are means ± S.D.

Condition	Sialic acid content (nmol per unit β-hexosaminidase)	Efflux (nmol/min per unit β-hexosaminidase)
Unincubated	259 ± 3	–
Control	267 ± 11	−0.5 ± 0.7
50 μM stearylamine	137 ± 28	4.1 ± 0.9

cystinosis, which is characterized by dysfunction of the cystine transporter, also lost stored cystine when incubated with stearylamine (Table II). These effects were not confined to lysosomal cystine storage as fibroblast lysosomes from an individual with inherited dysfunction of lysosomal sialic acid transport lost stored sialic acid upon exposure to stearylamine (Table II). Concentrations of stearylamine which were effective in treating isolated lysosomes killed cultured human fibroblasts within 2–3 h of exposure, making studies of intact cells impractical.

Discussion

In our studies, stearylamine caused the rapid release of cystine from lysosomes derived from rat liver, cultured human fibroblasts, and cultured cystinotic fibroblasts. While divalent and polyvalent cations also stimulate cystine efflux from normal lysosomes, they have no effect on efflux from cystinotic lysosomes [9]. Thus stearylamine appears to have a different mechanism of action from other cations. Since stearylamine is a weakly basic amine with a long chain length, it may be functioning as a lysosomatropic detergent. This mechanism of action has been suggested for similarly constructed compounds such as *N*-dodecylimidazide which are lipid soluble when unprotonated and then accumulate at low lysosomal pH in their charged forms [25]. Interestingly, these agents also may induce lysosomal cystine efflux [26,27]. Lysosomes incubated with stearylamine did not become readily alkalinized as might be expected with concentration of a weak base arguing against a lysosomatropic mechanism of action. Significant cytotoxic effects were observed with stearylamine. This has been noted with other putative lysosomatropic detergents and it has been suggested that these substances have non-lysosomal mechanisms of cytotoxicity [26,27].

Although lysosomal accumulation of stearylamine has not been demonstrated, the evidence does suggest that the effects of stearylamine on the lysosomal membrane are mediated through a detergent action. Stearylamine has no effects below its critical micellar concentrations and at lower relative protein concentrations lysosomes are disrupted. It is possible that at lower concentrations stearylamine inserts into the lysosomal membrane specifically permeabilizing it to substances such as cystine and sialic acid. Under these circumstances stearylamine may change membrane fluidity or change the specificity of transporters as has been proposed to account for the effects of increased temperature on lysosomal cystine efflux [28]. Other possible mechanisms are solubilization or disruption of selective membrane components. Analysis by SDS-PAGE to screen for solubilization of membrane proteins was inconclusive. However, since presumably only a small percentage of membrane proteins, if any, could be solubilized without disrupting membrane integrity, these findings are not surprising. As might be predicted, at unfavorable stearylamine/protein ratios, lysosomes are disrupted presumably due to membrane solubilization. Under controlled conditions, the membrane continues to function as a barrier to hydrolases and protons while allowing passage of cystine and sialic acid. Considering the size of the hydrogen ion, it is not clear why stearylamine does not induce a more severe proton leak.

The toxicity of stearylamine indicates that it is useful only under specific experimental conditions. Thus, although it depletes lysosomes with defective sialic acid transport of their sialic acid, stearylamine has no apparent role as an in vivo therapeutic agent. However, a similar acting but better tolerated agent could be of value in the treatment of disorders of lysosomal transport.

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References

1 Pisoni, R.L., Thoene, J.G. and Christensen, H.N. (1985) J. Biol. Chem. 260, 4791–4798.
2 Pisoni, R.L., Flickinger, K.S., Thoene, J.G. and Christensen, H.N. (1987) J. Biol. Chem. 262, 6010–6017.
3 Bernar, J., Tietze, F., Kohn, L.D., Bernardini, I., Harper, G.S., Grollman, E.F. and Gahl, W.A. (1986) J. Biol. Chem. 261, 17017–17112.
4 Gahl, W.A., Tietze, F., Bashan, N., Steinherz, R. and Schulman, J.D. (1982) J. Biol. Chem. 257, 9570–9575.
5 Gahl, W.A., Bashan, N., Tietze, F., Bernardini, I. and Schulman, J.D. (1982) Science 217, 1263–1265.
6 Jonas, A.J., Smith, M.L. and Schneider, J.A. (1982) J. Biol. Chem. 257, 13185–13188.
7 Jonas, A.J., Smith, M.L., Allison, W.S., Laikind, P.K., Greene, A.A. and Schneider, J.A. (1983) J. Biol. Chem. 258, 11727–11730.
8 Jonas, A.J. (1986) Biochem. J. 236, 671–677.
9 Jonas, A.J., Symons, L.J. and Speller, R.J. (1987) J. Biol. Chem. 262, 16391–16393.
10 Docherty, K., Brenchley, G. and Hales, C.N. (1979) Biochem. Soc. Trans. 7, 364–366.
11 Docherty, K., Brenchley, G. and Hales, C.N. (1979) Biochem. J. 178, 361–366.
12 Docherty, K. and Hales, C.N. (1979) FEBS Lett. 106, 145–148.
13 Maguire, G.A., Docherty, K. and Hales, C.N. (1983) Biochem. J. 212, 211–218.
14 Renlund, M., Tietze, F. and Gahl, W.A. (1986) Science 232, 759–762.
15 Jonas, A.J. (1986) Biochem. Biophys. Res. Commun. 137, 175–181.
16 Jonas, A.J., Speller, R.J., Conrad, P.B. and Dubinsky, W.P. (1989) J. Biol. Chem. 264, 4953–4956.
17 Jonas, A.J., Greene, A.A., Smith, M.L. and Schneider, J.A. (1982) Proc. Natl. Acad. Sci. USA 79, 4442–4445.
18 Barrett, A.J. (1972) in Lysosomes, A Laboratory Handbook (Dingle, J.T., ed.), pp. 46–126, American Elsevier, New York.
19 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
20 Chattopadhyay, A. and London, E. (1984) Anal. Biochem. 139, 408–412.
21 Murayama, J.-I., Tomita, T., Tsuji, A. and Hamada, A. (1976) Anal. Biochem. 73, 535–538.
22 Oshima, R.G., Willis, R.C., Furlong, C.E. and Schneider, J.A. (1974) J. Biol. Chem. 249, 6033–6039.
23 Baccino, F.M. and Zuretti, M.F. (1975) Biochem. J. 146, 97–108.
24 Okhuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA 75, 3327–3331.
25 Wilson, P.D., Firestone, R.A. and Lenard, J. (1987) J. Cell Biol. 104, 1223–1227.
26 Forster, S., Scarlett, L. and Lloyd, J.B. (1987) Biochim. Biophys. Acta 924, 452–457.
27 Forster, S., Scarlett, L. and Lloyd, J.B. (1988) Biochem. Soc. Trans. 16, 43–44.
28 Lemons, R.M., Pisoni, R.L., Christenson, H.N. and Thoene, J.G. (1986) Biochim. Biophys. Acta 884, 429–434.