The permeability of lysosomes to sugars

Effect of diethylstilbestrol on the osmotic activation of lysosomes induced by glucose

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We have investigated the effect on the osmotic activation of rat liver lysosomes, by glucose penetration, of different substances known to inhibit the glucose transport through the plasma membrane. Diethylstilbestrol is the most efficient, particularly when purified lysosomes are used. It has no effect on osmotic activation induced by hypo-osmotic sucrose or by iso-osmotic KCl. It is proposed that diethylstilbestrol reacts with specific sites involved in the glucose translocation through the lysosomal membrane. These sites could not be identified by binding experiments, presumably owing to the considerable unspecific binding of the compound to the membrane.

INTRODUCTION

Glucose and other monosaccharides can cross the lysosomal membrane. Until now, it has not been clearly established whether the translocation through the membrane results from a passive diffusion (Bird *et al.*, 1987) or involves a carrier (Maguire *et al.*, 1988). A study of the inhibition of the process by substances that specifically block the carrier-mediated transfer of glucose through the plasma membrane may provide further information on the problem. Some results were presented by Docherty *et al.* (1979) showing that phlorrhizin and cytochalasin B prevent, to some extent, glucose entry into lysosomes. However, the concentrations used by those authors were rather high; at such high levels, these substances could not be specific inhibitors of glucose translocation (Owen & Solomon, 1972).

On investigating the effect on the osmotic activation of lysosomes, by glucose penetration, of different substances known to inhibit the glucose transport through the plasma membrane, we found that diethylstilbestrol was extremely efficient. The low concentration (0.1–1 μM) of the substance required to cause a significant inhibition strongly suggests that the compound specifically reacts with a carrier molecule or with micro-domains of the membrane where the translocation takes place.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing about 200 g. Liver total mitochondria (ML fraction) were prepared as described by de Duve *et al.* (1955). Purified preparations of lysosomes were obtained by free-flow electrophoresis by the procedure described by Stahn *et al.* (1970), with an Elphor Vap 22 apparatus (Bender and Hobein, Munich, Germany). Lysosomal membranes were obtained by hypo-osmotic treatment of lysosomes purified by centrifugation in a metrizamide gradient as described by Wattiaux *et al.* (1978).

Permeability to glucose was assessed by the osmotic-

protection method of Lloyd (1969). The lysosome preparation was incubated at 25 °C in a medium containing 5 mM-Tris/HCl, pH 7.4, 0.25 M-glucose and 1 % dimethyl sulphoxide in a volume of 1 ml in the absence or presence of the compound to be tested. After 0 and 10 min, samples were withdrawn for assays of free and total activity of *N*-acetylglucosaminidase or cathepsin C.

For *N*-acetylglucosaminidase measurement, the lysosome preparation was incubated at 37 °C in a total volume of 1 ml containing 4 mM-*p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside, 0.1 M-citrate buffer, pH 5, and 0.25 M-sucrose in the absence (free activity) or presence (total activity) of 0.1 % Triton X-100. The assay was terminated after 10 min by adding 1.5 ml of 2.75 % (w/v) trichloroacetic acid. The released *p*-nitrophenol was determined by colorimetry as described by Vaes (1966). For assay of cathepsin C, the granules were incubated at 37 °C in a volume of 0.5 ml containing 0.25 mM-glycyl-L-arginine 2-naphthylamide, 50 mM-acetate buffer, pH 5, 5 mM-thioethylamine hydrochloride and 0.25 M-sucrose in the absence (free activity) or presence (total activity) of 0.05 % Triton X-100. The reaction was stopped by addition of 1 ml of 50 mM-glycine buffer, pH 10.5, containing 5 mM-EDTA and 0.5 % Triton X-100. The liberated naphthylamine was determined by measuring the fluorescence (excitation at 335 nm, emission at 415 nm) in a SPF 500 Aminco spectrofluorimeter. Proteins was also measured by the method of Lowry *et al.* (1951).

Binding of diethylstilbestrol to lysosomes was assessed by keeping lysosomal membranes or intact lysosomes for 10 min at 25 °C in a medium containing 5 mM-Tris/HCl, pH 7.5, 0.25 M-glucose and [³H]diethylstilbestrol (Amersham International, Amersham, Bucks., U.K.). After that, the mixture was centrifuged in a Beckman Airfuge for 20 min, the supernatant was withdrawn, and the bottom of the tube containing the pellet was cut off and dropped in a vial containing 4 ml of a liquid-scintillation cocktail (Aqualuma Lumac, Landgraaf, The Nether-

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lands). The radioactivity was measured in a Beckman β counter. The same procedure was applied to preparations devoid of lysosomes, and the radioactivity was subtracted from the values obtained in presence of the granules.

RESULTS

We found that several compounds known to inhibit glucose transport through the plasma membrane also inhibit the osmotic disruption of lysosomes by glucose, but at very different concentrations. This is exemplified by results recorded in Fig. 1, showing the response of lysosomes to glucose in presence of various concentrations of phlorrhizin, cytochalasin B, phloretin and diethylstilbestrol. It is obvious that diethylstilbestrol is markedly more potent. The concentration required to cause 50% inhibition in the assay conditions is 500 μ M for phlorrhizin, 125 μ M for cytochalasin B, 40 μ M for phloretin and 8 μ M for diethylstilbestrol. Diethylstilbestrol does not prevent osmotic disruption of lysosomes by hypo-osmotic media or by iso-osmotic KCl (Fig. 2).

Experiments whose results are presented here were performed with a total mitochondrial fraction. Some of them were repeated with lysosomes purified by free-flow electrophoresis. The amount of lysosomes that can be prepared by that method is small. Therefore, to perform each activation experiment with only one granule preparation, we used a spectrofluorimetric determination of cathepsin C that requires markedly less granules than for the *N*-acetylglucosaminidase measurement. The main difference observed was that the concentrations of diethylstilbestrol required to prevent osmotic disruption of lysosomes were markedly lower than with the mitochondrial fraction, as illustrated in Fig. 3. It shows the osmotic activation of purified lysosomes by glucose in presence of various concentrations of diethylstilbestrol,

Fig. 1. Free *N*-acetylglucosaminidase activity of ML fraction of rat liver after incubation at 25 °C in 0.25 M-glucose

ML fraction was incubated at 25 °C for 10 min in 0.25 M-glucose/5 mM-Tris/HCl buffer, pH 7.4, in the presence of diethylstilbestrol (Δ), phloretin ($\bullet$), cytochalasin B ($\blacktriangle$) or phlorrhizin ($\circ$). After that, free and total *N*-acetylglucosaminidase activities were measured. Ordinate: relative free activity (increase in free activity in the presence of the compound/increase in free activity in the absence of the compound). Abscissa: logarithm of the compound concentration. The free activity (as a percentage of total activity) at the beginning and end of the 10 min incubation period was respectively 12.9 and 41.4.

Fig. 2. Effect of diethylstilbestrol on osmotic activation of lysosomes by hypo-osmoticity or by 0.15 M-KCl

The granules were kept for 10 min at 0 °C in sucrose solutions of various concentrations (a) or for increasing times at 25 °C in 0.15 M-KCl (b), in the absence ($\bullet$) or presence ($\circ$) of 10 μ M-diethylstilbestrol. After that, free and total activities of *N*-acetylglucosaminidase were measured.

phloretin and cytochalasin B; 50% inhibition of increase of cathepsin C free activity is obtained at 0.4 μ M-diethylstilbestrol. The same effect required 25 times more phloretin and 160 times more cytochalasin B. It is also shown that, when diethylstilbestrol and phloretin are at too high a concentration, they induce a destabilization of the lysosomes, as indicated by the increase of cathepsin C free activity.

LeFevre (1959) compared agents closely related to diethylstilbestrol as inhibitors of glucose transport through red-cell membranes. We have tested these substances on lysosomes. Fig. 4 illustrates their inhibitory effect on the osmotic disruption of these organelles by glucose, and also their potency ratings as reciprocals of concentration (mM) producing 50% inhibition together with values obtained by LeFevre (1959) for red blood cells. The similarity between the two sets of results is evident.

Fig. 3. Free cathepsin C activity of lysosomes purified by free-flow electrophoresis after incubation at 25 °C in 0.25 M-glucose

Purified lysosomes were incubated at 25 °C for 10 min in 0.25 M-glucose/5 mM-Tris/HCl buffer, pH 7.4, in the presence of diethylstilbestrol (●), phloretin (○) or cytochalasin B (▲). After that, free and total cathepsin C activities were measured. Ordinate: relative free activity (increase in free activity in the presence of the compound/increase of free activity in the absence of the compound). Abscissa: logarithm of the compound concentration. The free activity (as a percentage of total activity) at the beginning and end of the 10 min incubation period was respectively 22.0 and 50.9.

To investigate whether diethylstilbestrol could be bound to lysosomes we incubated purified lysosomal membranes with [3 H]diethylstilbestrol and determined to what extent radioactivity becomes sedimentable after pelleting of the membranes. [3 H]Diethylstilbestrol binds to lysosomal membranes in an amount linearly related to its concentration and to the membrane concentration in the medium. No saturation is observed in the concentration range that we used (3 nM–5 μ M). It is obvious that the inhibitor effect of diethylstilbestrol on glucose transfer through the lysosomal membrane is observed near the lower end of the concentration range of the compound. Similar results were obtained with intact lysosomes purified by free-flow electrophoresis or by centrifugation in a metrizamide gradient.

DISCUSSION

Diethylstilbestrol markedly inhibits the osmotic disruption of lysosomes incubated in 0.25 M-glucose. The fact that the compound does not protect lysosomes against the osmotic stress caused by hypo-osmotic solutions or by iso-osmotic KCl strongly suggests that it does not act by stabilizing the lysosomal membrane, but by impeding glucose penetration in the organelles.

How does diethylstilbestrol prevent glucose transfer through the lysosomal membrane? Melchior & Czech (1979) have shown that carrier-mediated transport of glucose in reconstituted vesicles markedly depends on fluidity of the phospholipid bilayer. On the other hand, it is well known that steroids and related compounds can change the physical state of lipid bilayers and affect their permeability. As diethylstilbestrol is able to bind to the lysosomal membrane, it could modify its permeability to

Fig. 4. Free *N*-acetylglucosaminidase activity of ML fraction of rat liver after incubation at 25 °C in 0.25 M-glucose: effect of substances related to diethylstilbestrol

ML fraction was incubated at 25 °C for 10 min in 0.25 M-glucose/5 mM-Tris/HCl buffer, pH 7.4, in the presence of diethylstilbestrol (●), hexestrol (○), dienestrol (▲) or naringenin (Δ). After that, free and total *N*-acetylglucosaminidase activities were measured. Ordinate: relative free activity (increase in free activity in the presence of the compound/increase in free activity in the absence of the compound). Abscissa: logarithm of the compound concentration. The free activity (as a percentage of total activity) at the beginning and end of the 10 min incubation period was respectively 12.5 and 56.8. Below are given reciprocals of concentrations (in mM) that produce 50% inhibition, together with the values obtained by LeFevre (1959) for red blood cells. The numbers in parentheses give this values as percentages of the values for diethylstilbestrol.

	Lysosomes	Red blood cells
Diethylstilbestrol	320 (100)	160 (100)
Hexestrol	125 (42)	70 (44)
Dienestrol	77 (24)	15 (9.3)
Naringenin	8 (2.5)	7 (4.3)

glucose by changing its fluidity. However, such a mechanism is difficult to reconcile with the low diethylstilbestrol concentration required to inhibit glucose penetration in lysosomes; 1 μ M-diethylstilbestrol causes a strong inhibition of glucose transfer through the lysosomal membrane. At such a concentration 3 nmol of the compound is bound/mg of lysosomal membrane protein, corresponding to about 10 nmol/ μ mol of phospholipid (Basu *et al.*, 1986). It is improbable that, at such a concentration, diethylstilbestrol could perturb the physical state of the lysosomal membrane lipid bilayer and, particularly, decrease passive diffusion of molecules through it. The most plausible explanation is that diethylstilbestrol reacts with specific sites involved in glucose transport through the lysosomal membrane but that could not be identified by binding experiments, owing to

the considerable unspecific binding of the compound to the membrane. We think that such an unspecific binding of diethylstilbestrol occurs on membranes of other organelles, owing to their lipid content (Wheeler & Hinkle, 1981). It probably explains the difference in concentration of that compound required to obtain the same effect depending on whether a ML fraction or purified lysosomes are used. The major components (in mass) of the ML fraction are mitochondria. The binding of diethylstilbestrol to these organelles presumably markedly decreases the free molecule concentration in the medium.

Our observations are similar to those made many years ago by LeFevre (1959) and LeFevre & Marshall (1959) on glucose transport in red blood cells that show that diethylstilbestrol and related inhibitors of sugar transport bind to the red-cell membrane throughout a wide concentration range extending far above that at which inhibition of sugar transport is maximal. They bring additional arguments in favour of the presence of a glucose transporter in the lysosomal membrane.

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