Lysosomal glycogen storage induced by Acarbose, a 1,4- α -glucosidase inhibitor

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The 1,4- α -glucosidase inhibitor, Acarbose, when injected intraperitoneally disturbs liver lysosome metabolism, causing distinct and persistent inhibition of the enzymes and acute disturbances of lysosomal glycogen metabolism. A feedback control mechanism appears to operate, affecting cytosolic carbohydrate metabolism. A model is suggested for the adult form of lysosomal storage disease. The biochemical effects closely resemble those occurring in glycogenosis type II (Pompe's disease), and these have been confirmed by electron microscopy.

It is now well established that in the liver, and some other tissues, a portion of the cellular content of glycogen is associated with the lysosomes (Geddes & Stratton, 1977a; Knecht & Hernandez, 1978; Brown *et al.*, 1978; Rybicka, 1981). Further, this glycogen may be distinguished from that in the cytosol, to various extents, by size, structure and incorporation of radioactive precursors of glycogen synthesis (Geddes & Stratton, 1977a,b; Geddes *et al.*, 1977a,b). The size distribution of liver glycogen was shown to be distinctly affected by the anti-inflammatory drugs salicylate and indomethacin (Geddes *et al.*, 1983). By measurement of the incorporation of radioactive glucose into glycogen, salicylate was shown to have a depressing effect on overall liver glycogen metabolism. These effects appear to arise from the stabilization of the lysosome by the drugs.

The genetic lack of 1,4- α -glucosidase (EC 3.2.1.3) results in Pompe's disease (glycogenosis type II), with accumulation of glycogen particles in the lysosomal compartment. The incorporation, via liposomes, of purified anti-1,4- α -glucosidase antibodies into liver lysosomes causes a distinct decrease in 1,4- α -glucosidase activity and in the content of high- M_r glycogen. These changes are increased by prolonged liposomal antibody treatment and suggest that a possible feedback control mechanism operates in the incorporation of glycogen into lysosomes (Geddes *et al.*, 1983).

Other reports have described how intraperitoneal injections of the 1,4- α -glucosidase inhibitor Acarbose (Schmidt *et al.*, 1977; Puls *et al.*, 1980; Padilla *et al.*, 1981) experimentally induced glycogenosis as assessed histochemically and cytochemically (Lüllman-Rauch, 1981, 1982). The portion of hepatic glycogen that is normally found in the

lysosomal compartment is not degraded, because of the enzyme inhibition, and accumulates (Lüllman-Rauch, 1981). [Acarbose is a pseudotetra-saccharide composed of a maltose unit with a free reducing end linked by a β -amylase-sensitive bond to an amino sugar *N*-linked to a cyclitol (Lüllman-Rauch, 1981).]

Since lysosomal glycogen is relatively easily distinguishable from its cytosolic counterpart, particularly by size (Geddes & Stratton, 1977a,b), we decided to assess the biochemical effects of Acarbose. Lysosomal glycogen, which accounts for approx. 10% of the total cellular glycogen (Geddes & Stratton, 1977a), is distinguished from cytosolic glycogen by a predominance of high- M_r (up to 1000×10^6) molecules (Geddes & Stratton, 1977a; Geddes *et al.*, 1977a, 1983; Calder & Geddes, 1983). In addition, large glycogen molecules are composed of relatively small spheres of the polysaccharide (M_r 10×10^6) covalently linked to a protein backbone, which is linked by disulphide bonding to other identical protein-glycogen molecules (Chee & Geddes, 1977; Geddes & Stratton, 1977b; Geddes *et al.*, 1977b). It is also noteworthy that large glycogen molecules of similar structure have been reported in skeletal muscle (Calder & Geddes, 1985), and that retinal glycogen has been reported (Aon & Curtino, 1984) to have a high protein content.

Materials and methods

A sample of Acarbose (Bay g 5421) was generously supplied by Professor R. Lüllman-Rauch (Department of Anatomy, University of Kiel, Federal Republic of Germany). In all cases male Wistar rats (170 ± 5 g) were used. The animals

were maintained under standard animal-house conditions (14h light, 10h dark, $20 \pm 1^\circ\text{C}$). Acarbose (400mg/kg) dissolved in 0.9% NaCl/0.1 M-sodium phosphate buffer (pH 7.2) was administered by intraperitoneal injection, and the rats were killed either 24h or 5 days later. Control rats received the same amount of this phosphate-buffered saline. Half of the animals were starved for 17h before death, to deplete the hepatocytes of cytoplasmic glycogen. All experiments were performed on sets of randomly selected animal pairs.

Glycogen was isolated from rat livers by a cold-water extraction method as described previously (Geddes & Stratton, 1977a,b), with care being taken to minimize the rapid non-uniform degradation of hepatic glycogen *post mortem* (Geddes & Rapson, 1973). Fractions were obtained by density-gradient centrifugation in sucrose and their average M_r values calculated as described previously (Geddes *et al.*, 1977a). Concentrations were measured by CaCl_2 -enhanced absorption of the glycogen-iodine/iodide complex (Krisman, 1962). Glycogen content of whole liver was determined by the method of Kemp & van Heijningen (1954).

Acid 1,4- α -glucosidase activity was assayed at pH 4.0 with 4-methylumbelliferyl 1- α -D-glycopyranoside (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) as substrate (Willcox & Ratray, 1979). Assay conditions were: 0.1 ml of 2 mM-umbelliferyl substrate in citrate/phosphate buffer (pH 4.0) incubated with 0.1 ml of homogenate at 37°C for 10 min; the reaction was terminated by addition of 1 ml of 0.5 M-glycine/NaOH buffer (pH 10.4). The released 4-methylumbelliferone was measured by fluorimetry (excitation 358 nm, emission 448 nm). Blanks (0.1 ml of buffer substituted for the homogenate) were subtracted from all measurements. Other lysosomal enzymes were assayed similarly with umbelliferyl substrates.

Lysosomal glycogen was prepared by the rapid differential-centrifugation technique of Geddes & Stratton (1977a), followed by a similar cold-water extraction method used to isolate whole liver glycogen. However, to increase the reproducibility of lysosomal preparations, a constant-velocity automatic homogenizer of the type described by Dingle & Barrett (1969) was constructed. Higher and more consistent yields of intact lysosomes were obtained in the lysosome-enriched fractions by this technique. Lysosomal and whole-liver glycogen contents were determined on the same livers.

For electron-microscopic studies, small blocks (approx. 2 mm square) of liver tissue were fixed in 3% (v/v) glutaraldehyde in 0.1 M-sodium phosphate buffer, pH 7.2. They were then post-fixed with 2% OsO_4 , dehydrated with ethanol and embedded in Epon 812. Ultrathin sections were stained with

lead citrate and uranyl acetate and viewed under a Philips EM 200 electron microscope. The fixed sections were later treated with 10% (v/v) H_2O_2 , followed by 10% (v/v) HIO_4 , and then with saturated KIO_4 to remove the osmium. This was necessary, as in the thiosemicarbazide (TSC)-silver proteinate method used for glycogen detection the OsO_4 interferes with the specificity of the technique (Hanker *et al.*, 1966). It is best to employ post-fixation with osmium, followed by its removal, as this often produced better morphological preservation than did aldehyde fixation alone.

The TSC-proteinate method was used, as it has high specificity for periodic acid-Schiff-positive substances (Thiery, 1967), the glycogen is well stained and the reaction product with silver proteinate is very fine, permitting localization with high resolution. The method involves washing free-floating sections with aq. 1% (v/v) HIO_4 , followed by two rapid washes in distilled water. The sections were then treated with 0.2% trichloroacetic acid in 20% acetic acid. These were then washed rapidly with 10% acetic acid (twice) and then successively with 10% acetic acid (twice, 15 min), 5% acetic acid (twice, 5 min), 1% acetic acid (twice, 5 min) and then distilled water. The sections were finally treated with 1% silver proteinate at room temperature in the dark and washed in several changes of distilled water before mounting on inert gold grids.

Results and discussion

Fig. 1 shows the effect on the M_r distribution of liver glycogen of a single-dose Acarbose treatment over 1 and 5 days. The initial dramatic decrease in the relative amounts of medium- and high- M_r glycogen is beginning to be reversed after 5 days. The Acarbose dosage (400 mg/kg) is well below toxic values (Schlüter, 1981). Further, the serum concentration has a half-life of approx. 20 min after intravenous injection, with most of the Acarbose being excreted via the urine within a few hours (Pütter *et al.*, 1982). It is therefore surprising to find such persistent changes in the glycogen distributions after single Acarbose injections assessed many hours after treatment. Clearly the Acarbose must be concentrating in the lysosomal compartment, as implied by the observations of Lüllman-Rauch (1981, 1982).

Fig. 2 shows that the result of Acarbose treatment on the glycogen isolated from the lysosomal compartment is an initial increase in the proportion of the high- M_r material, which is still obvious even 5 days after the Acarbose treatment. Clearly the inhibition of 1,4- α -glucosidase activity by Acarbose slows the hydrolytic degradation of high- M_r glycogen within the lysosomal compart-

ment, whereas the extralysosomal storage of this material decreases dramatically (Fig. 1). The quantitative effects in the lysosomal compartment are detailed in the Tables.

Table 1 shows that the relative distribution changes observed in Fig. 1 are paralleled by decreases (30%) in the overall glycogen content of the liver, but as in Fig. 2 the lysosomal glycogen

content shows a small percentage increase. Again the persistence of the effect is shown by the results 5 days after the Acarbose treatment. In animals starved for 17h before death the increased importance of the lysosomal compartment and the duration of the effect are especially obvious.

Fig. 1. *M_r* distributions of whole-liver glycogens from Acarbose-treated and control rats

Rats that had been intraperitoneally injected with Acarbose 1 day (◇) or 5 days (□) before death are compared with untreated (control) animals (●). All results were averaged from three determinations, and were obtained by sucrose-density-gradient centrifugation (Geddes & Stratton, 1977a).

Fig. 2. *M_r* distributions of lysosomal glycogens from Acarbose-treated and control rats

Rats that had been intraperitoneally injected with Acarbose 1 day (◇) or 5 days (□) before death are compared with untreated (control) animals (●). All results were averaged from three determinations. Lysosomal glycogen was isolated by a modification of the method of Geddes & Stratton (1977a) and the distributions determined by sucrose-density-gradient centrifugation (Geddes & Stratton, 1977a).

Table 1. Glycogen content of whole liver after Acarbose treatment

‘Starved’ refers to a period of 17h before death. Control animals were treated in exactly the same manner and at the same time as the experimental group, except for the intraperitoneal injection of Acarbose (400mg/kg). Results are shown as means $\pm$ S.D. for preparations from sets of *n* animals. Total glycogen was measured by the method of Kemp & van Heijningen (1954) and lysosomal glycogen by a modification of the method of Geddes & Stratton (1977a).

Time between Acarbose treatment and death ...	Liver wt. (g)	Total glycogen (mg)	Glycogen content				
			1 day		5 days		
			Total glycogen (mg)	Lysosomal glycogen (% of total)	Total glycogen (mg)	Lysosomal glycogen (mg)	Lysosomal glycogen (% of total)
Control (<i>n</i> = 10)	8.6 $\pm$ 0.9	462 $\pm$ 37	33 $\pm$ 4	7.0	473 $\pm$ 38	30 $\pm$ 4	6.0
Acarbose (<i>n</i> = 8)	7.8 $\pm$ 0.7	317 $\pm$ 25	39 $\pm$ 5	12	329 $\pm$ 26	45 $\pm$ 5	14
Control, starved (<i>n</i> = 10)	5.3 $\pm$ 0.3	17 $\pm$ 1	13 $\pm$ 2	76	18 $\pm$ 1	14 $\pm$ 2	77
Acarbose, starved (<i>n</i> = 8)	5.6 $\pm$ 0.3	33 $\pm$ 3	16 $\pm$ 2	48	53 $\pm$ 4	34 $\pm$ 4	85

Table 2. *Changes of glycogen size distribution in the lysosomal compartment*

Data are presented as the change (%) in each size range compared with the same size range in controls (see Table 1 and Fig. 2). Results are means $\pm$ S.D. for ten separate preparations.

	$10^{-6} \times M_r$ range of lysosomal glycogen	Time between Acarbose treatment and death ...	Change in glycogen content relative to control	
			1 day	5 days
Normal	< 250		-34 ± 5	-15 ± 2
	250–500		$+25 \pm 4$	$+16 \pm 2$
	> 500		$+22 \pm 3$	$+7 \pm 1$
Starved	< 250		-12 ± 2	$+12 \pm 2$
	250–500		$+27 \pm 4$	-5 ± 1
	> 500		$+22 \pm 3$	-7 ± 1

Table 3. *Changes in 1,4- α -glucosidase activity in liver cells after Acarbose treatment*

Data are presented as the percentage increase or decrease relative to either control glucosidase activity (A) (Table 1), or control lysosomal glycogen content (B), or control values for various enzyme activities (C) (Geddes *et al.*, 1983). Control animals were treated identically with the experimental animals, except for the omission of Acarbose treatment. Results are means $\pm$ S.D. for *n* separate preparations.

Change in 1,4- α -glucosidase relative to:	Time between Acarbose treatment and death ...	Change (%)			
		1 day		5 days	
		Normal (n = 5)	Starved (n = 4)	Normal (n = 5)	Starved (n = 4)
(A) Total 1,4- α -glucosidase activity		-46 ± 11	-59 ± 11	-48 ± 8	-65 ± 8
(B) Lysosomal glycogen content		-55 ± 12	-67 ± 11	-65 ± 13	-85 ± 12
(C) Acid phosphatase		-28 ± 6	-36 ± 7	-62 ± 12	-48 ± 10
β -Glucuronidase		-12 ± 2	-49 ± 10	-33 ± 7	-20 ± 4
β -Galactosidase		-39 ± 5	-34 ± 7	-42 ± 8	$+8 \pm 2$

Although the persistence is very marked, the slow recovery from Acarbose treatment noted by Lüllman-Rauch (1981) may also be perceived. Table 2 shows that in well-fed animals the initial accumulation of high- M_r glycogen in the lysosomal compartment and loss of low- M_r glycogen is significantly modified after 5 days. In the starved animals the slow return to normality is also clear.

Table 3 illustrates the dramatic decrease in 1,4- α -glucosidase activity relative to other lysosomal enzymes and glycogen. Acarbose has a dramatic inhibiting effect, suggesting that it may be concentrated within the lysosome, thereby disturbing the normal lysosomal clearance of high- M_r glycogen (Geddes & Stratton, 1977a; Geddes *et al.*, 1983). However, this is paralleled by a decrease in the total hepatic glycogen content in well-fed animals. Thus these results show, in agreement with experiments where the lysosomal carbohydrate metabolism was disturbed by anti-1,4- α -glucosidase antibodies (Geddes *et al.*, 1983), that disturbance of normal liver lysosomal function caused

acute disturbance to the overall cellular carbohydrate metabolism. This indicates a sensitive feedback control mechanism on overall glycogen synthesis within the cell and the method by which glycogen is incorporated into the lysosomes. Iwamasa *et al.* (1980) noted that in various cells glycogen autophagy appeared to be related to lysosomal 1,4- α -glucosidase activity. Our results confirm this, but suggest that the process may be more complex. Like most lysosomal enzymes, 1,4- α -glucosidase activities vary as compared with other lysosomal enzymes (Table 3) (Geddes & Stratton, 1977a), but these activities also change with treatment (Acarbose, starvation), indicating that the activities of the other enzymes may also be subject to varying degrees of control.

We were able to confirm further our results by electron microscopy. Hepatocytes of control and control-starved animals, injected with 0.9% NaCl (pH 7.2), contained very few lysosomes, and these were filled with an electron-dense matrix but no obvious glycogen particles. Control animals con-

tained numerous amounts of cytosolic glycogen both in its monogranular form and less frequently as rosette-like aggregates (Fig. 3a), where control-starved animals had very little cytoplasmic glycogen (Fig. 3b). In test animals killed 1 day after Acarbose injection, all hepatocytes (and the occasional Kupffer cell) contained numerous enlarged lysosomes measuring up to $2.5\mu\text{m}$ in diameter.

These vacuoles were filled with granular material identified histochemically as glycogen. Prolongation of survival time up to 5 days increased not only the number and size of lysosomes and the glycogen-storage phenomena, but also the quantity of cytosolic glycogen (Fig. 3c). In addition, Acarbose-treated starved rats seemed to be even more affected by the treatment (Fig. 3d).

Fig. 3. Electron micrographs of semithin Epon sections post-fixed with 2% OsO_4 and lightly stained with lead citrate and uranyl acetate

(a) Liver of a control rat; (b) liver of a control rat starved for 17 h before death; (c) liver of a rat treated with Acarbose 5 days previously; (d) liver of a rat treated with Acarbose 5 days previously and starved for 17 h before death. All micrographs are at the same magnification (approx. 31 350); the bar indicates a dimension of $1\mu\text{m}$. Abbreviation: m, mitochondrion.

In summary, although Acarbose dramatically affects lysosomal enzymic activity, its influence extends to the normal phosphorolytic pathway (Cohen, 1982), causing accumulation of low- M_r glycogen, as was observed with anti-(1,4- α -glucosidase) treatment (Geddes *et al.*, 1983). The effects in the lysosomal compartment are very marked and persistent (Tables 1 and 2), implying accumulation of the Acarbose in the lysosome. It is suggested that interference with 1,4- α -glucosidase activity by either antibody or Acarbose provides a model for the mild (adult) form of Pompe's disease (Engel *et al.*, 1973). The infantile form, with its massive accumulation of lysosomal glycogen and organelles which can reach up to 8 μ m in diameter (de Barsey, 1976; Hers, 1963), obviously overrides the feedback control mechanism described above and must be related to a separate genetic lesion.

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