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Rat Heart Perfusion as Model System for Enzyme Replacement Therapy in Glycogenosis Type II

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ABSTRACT. Cardiac failure and skeletal muscle weakness are the main clinical features of glycogenosis type II, a lysosomal storage disorder caused by acid α-glucosidase deficiency. In our study, we have investigated in a rat heart perfusion-recirculation system whether acid α-glucosidase can be taken up from the vascular system into cardiomyocytes. When rat hearts were perfused with mannose 6-phosphate-containing acid α-glucosidase purified from bovine testis, a 3- to 4-fold increase of enzyme activity was obtained. Perfusion with human placental acid α-glucosidase not containing the mannose 6-phosphate recognition marker did not have such an effect. The presence of bovine testis acid α-glucosidase in heart tissue was demonstrated by immunoblotting. Immunocytochemistry provides evidence for uptake of the exogenous enzyme in lysosomes of the cardiomyocytes. The relevance of these findings for enzyme therapy in glycogenosis type II is discussed. (*Pediatr Res* 28: 344–347, 1990)

Abbreviations

MU, 4-methylumbelliferone

Glycogenosis type II (Pompe's disease) is a lysosomal storage disorder characterized by deficiency of acid α-glucosidase (1, 2). The disease has an autosomal recessive mode of inheritance, and is clinically heterogeneous (3, 4). Patients with an infantile subtype of glycogenosis type II present with severe hypotonia, cardiomegaly, and moderate hepatomegaly. The life expectancy is from 1 to 2 yr. In late onset juvenile and adult variants, skeletal muscle weakness is usually the only clinical symptom, and respiratory failure is the major cause of death. The severity of symptoms appears to correlate closely with the extent of lysosomal glycogen storage and the level of residual acid α-glucosidase activity (5–8).

In the past 25 years, much effort has been devoted to the development of therapy for lysosomal storage diseases, but little progress has been made. At present, bone marrow transplantation attracts the most attention. In some cases, it seems to halt or delay the development of symptoms (9, 10). Bone marrow transplantation, however, appeared ineffectual for glycogenosis type II (9–12); in addition, earlier attempts at enzyme replacement therapy using acid α-glucosidase preparations from human placenta and *Aspergillus niger* have failed (13–15).

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It has been suggested that the effect of enzyme therapy can be improved by using cell surface receptors for efficient delivery of enzyme to the target tissues (16). One such receptor, recognized for its function in uptake of lysosomal enzymes with phosphorylated carbohydrate moieties, is the 270-kD mannose 6-phosphate receptor (17). This receptor is presently known to be identical to the IGF-II receptor (18), and has been demonstrated in a variety of cell types and tissues, including heart and skeletal muscle (19–22). Despite its apparent function in endocytosis of mannose 6-phosphate-containing lysosomal enzymes, the receptor has never been used as target in enzyme therapy (23, 24).

In previous studies, we have investigated the basic requirements for treatment of glycogenosis type II by receptor-mediated enzyme therapy (7, 8, 25, 26). We demonstrated that mannose 6-phosphate-containing acid α-glucosidases purified from bovine testis and human urine are taken up by cultured fibroblasts and skeletal muscle cells with a 100-fold greater efficiency than the enzyme species lacking this recognition marker. The phosphorylated enzymes were shown to be transported to the lysosomes and able to reverse completely the abundant glycogen storage in cells from even the most severely affected patients.

In our study, we used a rat heart perfusion system according to Langendorff to investigate whether acid α-glucosidase is transported from the vascular system across the endothelial barrier to the cardiomyocytes.

MATERIALS AND METHODS

Perfusion method. Hearts were obtained from 20-wk-old male Wistar rats under anesthesia with diethylether. They were mounted in a perfusion system according to Langendorff, and cannulated in the aorta (27, 28). Thus, the coronary vascular system was perfused selectively. Experiments were performed at 37°C, pH 7.4, using a modified Tyrode's buffer (128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, and 11 mM glucose, saturated with 95% O_2 and 5% CO_2). After an equilibration period of 10 min, in which heart contractility and flow stabilized, a recirculation-perfusion system was connected, containing 35 mL of Tyrode's buffer to which acid α-glucosidase was added in an amount equivalent to 40 μmol MU/h. Two enzyme preparations from different sources were used. A mannose 6-phosphate-rich form of acid α-glucosidase was purified from bovine testis (29). The sp act of this enzyme was 338 μmol MU/mg protein/h. Acid α-glucosidase lacking mannose 6-phosphate was purified from human placenta (30) and had a sp act of 276 μmol MU/mg protein/h. After 72 min, the recirculation system was disconnected, and the heart was perfused with the initial equilibration buffer for another 25 min. During perfusion, the contractility was monitored continuously, and samples were taken at regular intervals to measure

the acid α-glucosidase activity. At the end of the experiment, the heart was removed from the perfusion system and quickly divided into two halves. One part was embedded in Tissue-tek (Miles Research, Elkhart, IN) to perform immunocytochemistry. The other part was frozen in liquid nitrogen for biochemical assays.

Biochemical assays. Rat hearts were homogenized in 400 μL of distilled water with a Potter-Elvejhem homogenizer at 1300 rpm and subsequent sonication. Cell debris was removed by centrifugation at 15 000 × *g*. Acid α-glucosidase activities were measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside as described previously (31). Protein concentrations were determined by following the procedure of Lowry *et al.* (32).

Immunoassays. Antiserum against human placental acid α-glucosidase was raised in Wistar rats, and antiserum against bovine testis acid α-glucosidase was raised in Swiss mice, essentially as described before (30). Human placental as well as bovine testis acid α-glucosidase were recognized by rat Ig raised against the human enzyme. Cross-reaction with rat acid α-glucosidase did not occur. Also, the mouse Ig raised against the bovine acid α-glucosidase did not recognize the homologous enzyme from rat.

Rat heart homogenates were diluted to an acid α-glucosidase activity of 1 μmol MU/h/20 μL. Samples of this volume were incubated with 10 μL-serial dilutions of rat antiserum in PBS containing 1 mg/mL BSA or with PBS/BSA alone (as infinite antiserum dilution). Staphylococcus A membranes (10 μL of a 1:1 dilution in PBS/BSA) were added to precipitate immune complexes. Incubations were performed overnight at 4°C. The membranes were spun down at 15 000 × *g*, and acid α-glucosidase activities were determined in the supernatant and on the membranes.

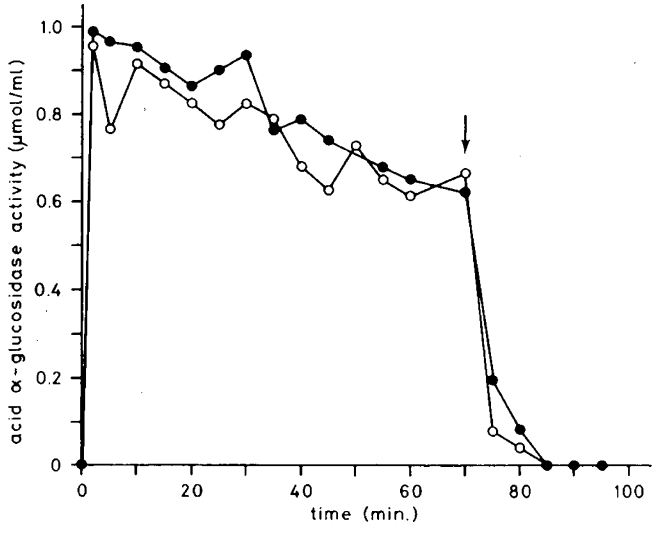

Fig. 1. Acid α-glucosidase activities in the perfusate. Enzyme perfusion was performed with purified bovine testis (●) or human placental (○) acid α-glucosidases (40 μmol MU/h in 35 mL Tyrode's buffer). After enzyme perfusion (*arrow*), the hearts were rinsed with Tyrode's buffer for an additional 25 min.

Table 1. *Acid α-glucosidase activities in rat hearts perfused with or without added enzyme*

Enzyme added to perfusion fluid	Enzyme activity in heart (nmol MU/mg protein/h)*
None (n = 8)	10.9 ± 1.3
Bovine testis (n = 5)	37.5 ± 7.0
Human placenta (n = 5)	11.7 ± 1.5

* Mean ± SD.

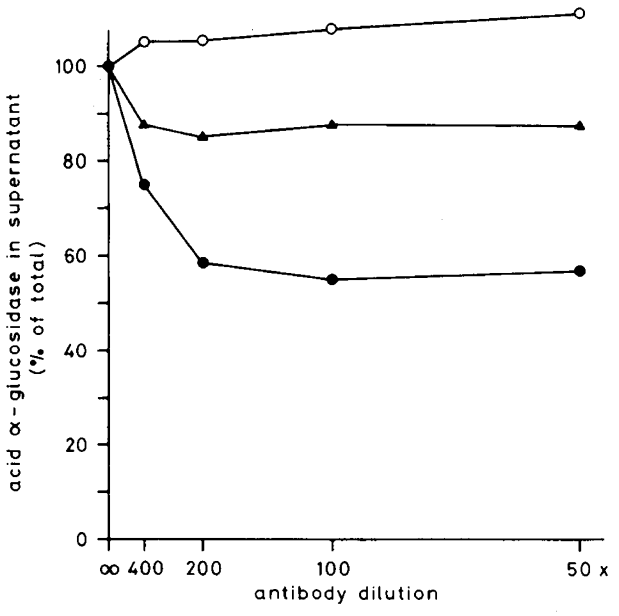

Fig. 2. Immunoprecipitation of bovine testis and human placental acid α-glucosidases. Rat heart homogenates were incubated with serial dilutions of rat antiserum against human placental acid α-glucosidase in combination with Staphylococcus A membranes. After overnight incubation at 4°C, membranes were spun down and acid α-glucosidase activities were measured in the supernatant. Perfusion without enzyme addition, ○; perfusion with bovine testis acid α-glucosidase, ●; perfusion with human placental acid α-glucosidase, ▲.

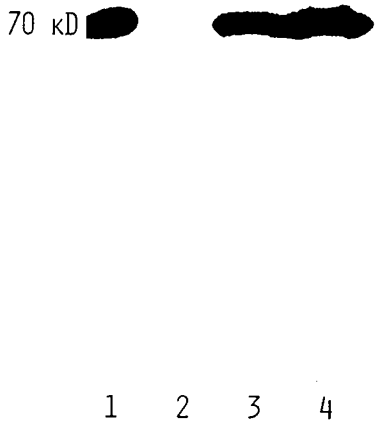

Fig. 3. Immunoblot analysis of bovine testis acid α-glucosidase in perfused rat heart. Bovine testis acid α-glucosidase was immunoprecipitated with rat antiserum against human placental acid α-glucosidase, subjected to PAGE, and immunoblotted. Enzyme protein was visualized with mouse antiserum against bovine testis acid α-glucosidase in combination with ^{125}I protein A. *Lane 1*: purified bovine testis acid α-glucosidase; *lane 2*: control heart; *lanes 3* and *4*: enzyme perfused rat hearts.

An immunocytochemical double labeling technique was used to localize bovine testis acid α-glucosidase and endogenous β-hexosaminidase together in the same section (33). For this purpose, frozen tissue sections (7 μm) were fixed in formaldehyde vapor and postfixed in 100% methanol. They were incubated with mouse antiserum against bovine testis acid α-glucosidase in combination with goat antiserum against mouse IgG conjugated to fluorescein (FITC, Nordic Pharmaceuticals, Ltd., Quebec, Canada), and with rabbit polyclonal antiserum against human β-hexosaminidase (30) in combination with goat antiserum against rabbit IgG conjugated to rhodamin (tetrarhodamine isothiocyanate, Nordic Pharmaceuticals, Ltd.).

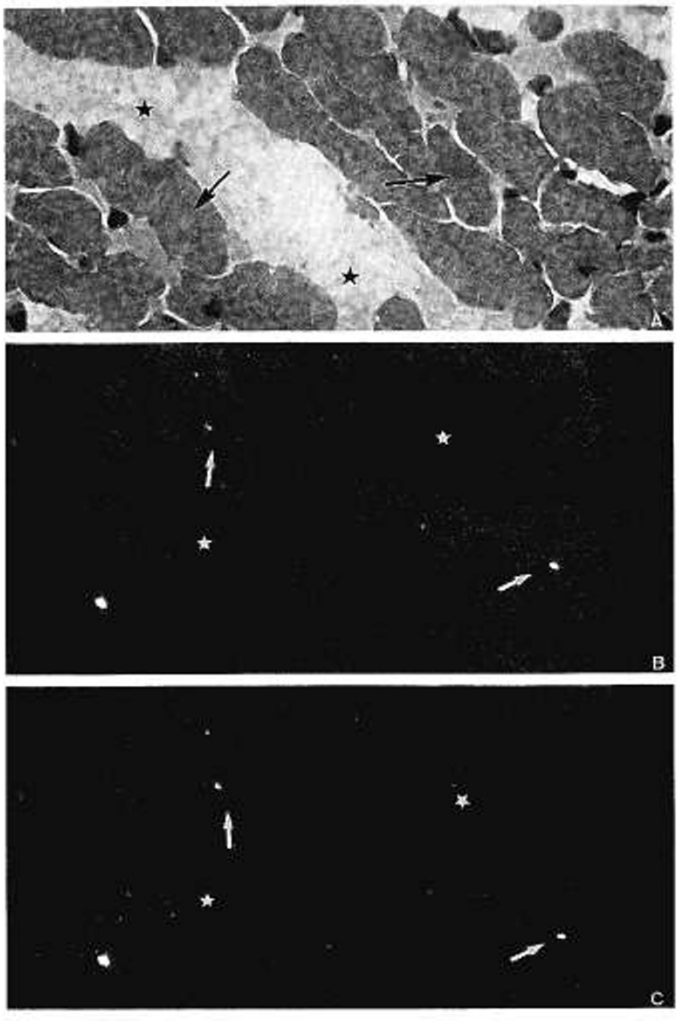

Fig. 4. Immunocytochemical localization of bovine testis acid α-glucosidase. *A*, rat heart section stained with hematoxylin-eosin: magnification 357.5×. *B*, section of rat heart after perfusion with bovine testis acid α-glucosidase. The section was incubated with mouse antiserum against bovine testis acid α-glucosidase in combination with goat antiserum against mouse IgG conjugated to fluorescein. *C*, the same section as in *B*, incubated with rabbit antiserum against human placental β-hexosaminidase in combination with goat antiserum against rabbit IgG conjugated to rhodamin to establish the location of lysosomes. The connective tissue areas in *A* (light) and *B* and *C* (dark) are marked with a *star*. The positions of the nuclei of some cardiomyocytes are indicated with *arrows*.

Immunoblotting. Rat antiserum against human placental acid α-glucosidase was added to 400-μL aliquots of rat heart homogenate (0.2 g wet wt/400 μL), together with 50 μL of protein A sepharose beads (1:1 diluted in PBS) to precipitate cross-reacting bovine testis acid α-glucosidase. Incubations were carried out overnight at 4°C. Nonspecifically bound proteins were removed by washing the beads four times with 1 mL of PBS. Bound acid α-glucosidase was dissolved by heating for 10 min at 90°C in sample buffer (125 mM Tris-HCl, pH 6.6, 2 M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Proteins were separated by size in a 10% polyacrylamide gel according to Laemmli (34) and blotted onto nitrocellulose filters as described by Towbin *et al.* (35). Bovine testis acid α-glucosidase was visualized with polyclonal mouse antiserum against bovine testis enzyme in combination with ^{125}I-labeled protein A (35).

RESULTS

To study uptake of acid α-glucosidase from the coronary vascular system into the cardiomyocytes, isolated rat hearts were perfused via a cannula inserted in the aorta. This perfusion system was designed for studying heart function (27, 28). After an equilibration period of 10 min, the heart was perfused with bovine testis or human placental acid α-glucosidase for 1 h. Samples of the perfusion fluid were taken at intervals to measure the stability of the enzyme in the fluid. Some inactivation of both enzymes was observed over the experimental period (Fig. 1). After 72 min, enzyme perfusion was stopped and the heart was rinsed for 25 min with the original equilibration buffer to clear the capillary bed. This resulted in a fast decline of enzyme activity in the perfusate. Hearts were taken off the perfusion apparatus only after the perfusate was completely devoid of acid α-glucosidase activity, and were quickly frozen.

Table 1 shows that a significantly higher acid α-glucosidase activity was measured in rat hearts perfused with bovine testis acid α-glucosidase than in those perfused without enzyme addition or with acid α-glucosidase isolated from human placenta. An immunologic test was carried out to investigate whether the increase of activity was actually due to uptake of bovine testis acid α-glucosidase rather than to stimulation of endogenous enzyme activity. For this experiment, we used an antiserum against human placental acid α-glucosidase, which binds over 90% of bovine testis as well as human placental acid α-glucosidases but does not cross-react with the homologous rat enzyme. With this antiserum, approximately 45% of the acid α-glucosidase activity was precipitable from homogenates of rat hearts perfused with bovine testis acid α-glucosidase. Much less activity was precipitable after perfusion with enzyme isolated from human placenta (Fig. 2).

Immunoblotting was performed to further define the origin and nature of the precipitable activity after perfusion with bovine testis acid α-glucosidase. For this purpose, mouse antibodies raised against the bovine enzyme were used, inasmuch as the rat antiserum used for immunoprecipitation did not react on immunoblots. Using this procedure, the administered 70-kD bovine testis enzyme (Fig. 3, *lane 1*) was demonstrated to be present in enzyme-perfused rat hearts (Fig. 3, *lanes 3* and *4*), but not in control hearts (Fig. 3, *lane 2*).

Finally, an attempt was made to localize the bovine testis acid α-glucosidase in thick, frozen sections of rat heart. In this case, the same mouse polyclonal antiserum was used as for immunoblotting. Few but distinct fluorescent spots were detected intracellularly in sections of enzyme perfused hearts (Fig. 4*B*), whereas control heart sections remained devoid of label. Endogenous β-hexosaminidase used as a lysosomal marker revealed an identical labeling pattern when its localization was determined in the same section (Fig. 4*C*). The number of lysosomes in these sections is typically low. No acid α-glucosidase was detectable in capillaries. Figure 4*A* shows for reference a similar rat heart section stained with hematoxylin and eosin.

DISCUSSION

Using a rat heart perfusion-recirculation system, evidence was obtained for uptake of mannose 6-phosphate-containing acid α-glucosidase from the coronary vascular system into the cardiomyocytes. Perfusion with bovine testis acid α-glucosidase resulted in a 3-fold increase of enzyme activity in the heart tissue. Inasmuch as the capillary bed is cleared after perfusion, the exogenous enzyme must have crossed the endothelial barrier. Indeed, the administered enzyme was not detectable with immunocytochemical methods in the capillaries in tissue sections. Instead, a punctate labeling of cardiomyocytes was observed in the proximity of the cell nucleus where lysosomes are localized and at the same spots as the lysosomal marker β-hexosaminidase. This suggests that acid α-glucosidase was taken up in the lysosomes of cardiomyocytes.

When immunoprecipitated from heart tissue, the 70-kD bovine testis acid α-glucosidase appeared unchanged compared with the purified enzyme that was added to the perfusion fluid. Taken

together, the observations indicate that acid α-glucosidase from bovine testis is transported across the endothelial cells into the lysosomes of cardiomyocytes. The specificity of this transport process is emphasized by the failure to find a significant increase of activity after perfusion with non-phosphorylated human placental acid α-glucosidase.

After perfusion with bovine testis acid α-glucosidase, an estimated 1–2% of the circulating enzyme was recovered in heart tissue. This figure compares to the efficiency of mannose 6-phosphate receptor-mediated uptake of bovine testis and human urine acid α-glucosidases by cultured fibroblasts and muscle cells. The recovery of these enzymes, added directly to the culture medium, was approximately 4% (7, 25). By contrast, the uptake of human placental acid α-glucosidase, not recognized by this receptor, was much less (0.04%) (25). Considering these figures, the uptake of bovine testis acid α-glucosidase from the coronary vascular system was actually a surprisingly efficient process. We had anticipated rapid internalization of the mannose 6-phosphate-containing bovine testis acid α-glucosidase by the cardiomyocytes, inasmuch as these cells express the 270-kD mannose 6-phosphate receptor on their surface (21, 22). However, the overall efficiency of the process suggests that there is also a rather efficient mechanism for transport of lysosomal enzymes across the capillary endothelium. Plasmalemmal vesicles may be involved in this transport. They are known to serve as transport vehicles for other macromolecules like albumin and transferrin (36, 37), and they also seem to be involved in the transport of lysosomal enzymes across the endothelium in skeletal muscle tissue (38). It is less likely that the 270-kD mannose 6-phosphate receptor takes part in transendothelial transport, inasmuch as endothelial cells were reported not to express this receptor at their surface (39).

From our results, one might speculate that mannose 6-phosphate-containing acid α-glucosidase will be more suitable for enzyme replacement therapy in glycogenosis type II than the enzymes previously used in clinical trials. The results of our study in the rat heart model system make it worthwhile to test the rationale for enzyme replacement therapy using mannose 6-phosphorylated enzymes in animal models, especially because the cloning of the genes coding for lysosomal enzymes has brought large scale production of these proteins within reach.

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