Bovine generalised glycogenosis type II

Uptake of lysosomal α-glucosidase by cultured skeletal muscle and reversal of glycogen accumulation

P.N. Di Marco, J.McC. Howell* and P.R. Dorling

School of Veterinary Studies, Murdoch University, Murdoch, 6150 Western Australia, Australia

Received 15 August 1985

Acid α-glucosidase (EC 3.2.1.20) was purified from fetal bovine muscle by affinity chromatography on concanavalin A and Sephadex G-100 and added to the culture medium of mature muscle cultures from animals affected by glycogenosis type II. The enzyme activity in homogenates of treated cultures was significantly increased within 4 h of the addition of enzyme, was maximal by 18 h and the internalised activity was stable for at least 48 h after the removal of the enzyme from the culture medium. The acid α-glucosidase activity was internalised with an uptake constant of 300 nM and a V_{max} of uptake of 133 nmol/h per mg protein. The glycogen concentration in affected cultures treated with exogenous acid α-glucosidase for 24 h had decreased by 20% and after a further 24 h of culture was comparable to the concentration observed in cultures from non-affected animals.

Glycogenosis type II *α-Glucosidase* *Enzyme uptake* *Pompe's disease* *Cultured muscle*

1. INTRODUCTION

Glycogenosis type II is a lysosomal storage disease which is caused by decreased or absent acid α-glucosidase activity [1]. The disease in humans presents in 3 forms which are distinguished by the age of onset and severity of changes [2]. The infantile and childhood forms are characterized by the total absence of enzyme activity and widespread accumulation of glycogen in most tissues, particularly skeletal and cardiac muscle, which are the most severely affected. We have recently described the disease in shorthorn cattle [3] and have shown that the biochemical and morphological abnormalities of skeletal muscle, that characterize the disease in vivo, are reproduced in culture [4]. There is no known treatment for this disease. Attempts have been made to treat the condition, in both humans and cattle, by injection of purified acid α-glucosidase [5–8] without any clinical improvement. Moreover, little if any enzyme activity accumulated in skeletal muscle [7,8]. This may have been either because the enzyme has a short half-life after injection [7,8] and thus the tissue was exposed to an insufficient amount of enzyme, or because skeletal muscle may not internalise the purified enzyme. The aim of this study was to test whether cultured skeletal muscle from cattle affected by glycogenosis type II takes up purified acid α-glucosidase and whether the internalised enzyme would reverse the abnormal accumulation of glycogen.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

Acid α-glucosidase (spec. act. 1.5 μmol/min per mg protein) was purified from fetal bovine skeletal muscle by affinity chromatography on concanavalin A and Sephadex G-100 as described [9]. The active material eluted from the Sephadex

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

G-100 was concentrated and washed over an Amicon PM 10 filter with 10 mM phosphate buffer, pH 6.8, and stored at −20°C until required. Bovine skeletal muscle was grown in culture as in [4]. On days 6–7, the cultures were incubated with purified acid α-glucosidase in 2 ml uptake medium (10 mM phosphate buffer, pH 6.8, containing 0.145 M NaCl, 9 mM $NaHCO_3$, 10% heat-inactivated fetal calf serum, 16.6 mM glucose, 2 mM glutamine, 1% penicillin-streptomycin-fungizone [M.A. Bioproducts], 0.05% phenol red). The cultures were incubated in humidified air containing 5% CO_2. Control cultures were incubated in uptake medium alone. Under these conditions, acid α-glucosidase activity was stable for at least 24 h, with 79% of the activity remaining after 66 h incubation. In other culture media [4] the enzyme had a half-life of 1.5 h. For the intracellular enzyme stability and glycogen content experiments, cultured muscle was incubated with purified enzyme as described above for 24 h, washed 3 times with phosphate-buffered saline, pH 7.4, and incubated in maintenance medium [4] for the remainder of the experiment. At the completion of the experiments, the cultures were washed 3 times with cold phosphate-buffered saline, pH 7.4, and harvested for acid α-glucosidase activity, protein and glycogen estimations as described in [4]. The results are expressed as the mean ± SE with the number of observations given in parentheses. In the figures, the SE is shown by vertical bars. Statistical analyses were performed using Student's unpaired *t*-test.

3. RESULTS AND DISCUSSION

Early clinical trials of enzyme-replacement therapy in glycogenosis type II resulted in no clinical improvement in the patients [1]. Although injected acid α-glucosidase activity was detected in liver and lysosomal glycogen appeared to be decreased in that organ, no enzyme activity or changes in glycogen concentration could be detected in muscle [6,7]. Similar results were reported for cattle [8]. From these studies however, it was not possible to conclude that the lack of enzyme in skeletal muscle was due to the inability of that tissue to take up the enzyme. The half-life of the injected acid α-glucosidase activity in the plasma was 10 min or less [7,8]. Thus the activities available for uptake by peripheral tissues, such as muscle, may have been too low.

Our studies suggest that cultured skeletal muscle from calves affected by glycogenosis type II takes up exogenous enzyme and that the uptake is both time and dose dependent (fig.1). The acid α-

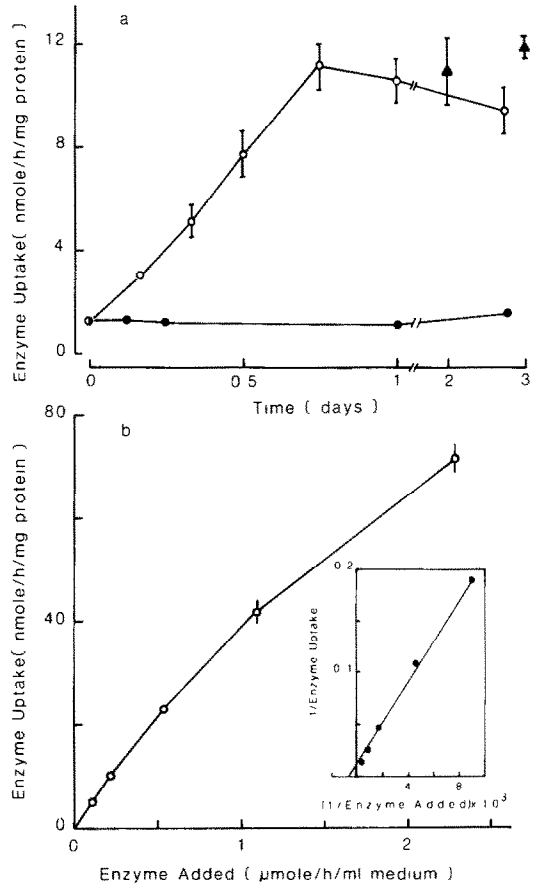

Fig.1. Uptake of lysosomal acid α-glucosidase by cultured skeletal muscle. (a) Time course of uptake and intracellular stability. Day 7 cultures from affected animals were incubated with 0.66 μmol/h per ml acid α-glucosidase (○—○). At the indicated times individual dishes were harvested for enzyme assay as described. Control cultures were incubated in medium without the enzyme (●—●). For the intracellular stability experiment (▲—▲), cultures were incubated with the enzyme for 24 h, then maintained in culture up to the times indicated. (b) Dose response. Day 6 cultures were incubated with acid α-glucosidase (spec. act. 1.5 μmol/min per mg protein) for 24 h then harvested for enzyme assay as described in the text. Each point is the mean of at least 3 determinations from individual dishes except for the controls in panel a which are the means of duplicate determinations.

glucosidase activity in the homogenates of affected cultures incubated with purified enzyme was significantly increased ($p < 0.05$) within 4 h of addition of the enzyme and was maximal within 18–24 h (fig.1a). There was no decrease in the acid α-glucosidase activity in homogenates of treated cultures for at least 48 h after the removal of the enzyme from the culture medium (fig.1a). This observation suggests that the internalised enzyme was stable which is in agreement with published data on the intracellular stability and turnover of acid α-glucosidase activity both in vivo and in vitro [6,10,11]. At high concentrations of enzyme, the uptake became saturating (fig.1b) and the activity of acid α-glucosidase in the homogenates of treated cultures increased to about 70% of the activity in muscle cultures from non-affected animals [4]. A Lineweaver-Burk plot (fig.1b, inset) of the uptake data gave an apparent uptake constant of 300 nM, assuming an M_r of 107000 for the bovine enzyme [8,12], and a V_{max} of uptake of 133 nmol/h per mg protein.

In fibroblasts, uptake of acid hydrolases is dependent on either a high-efficiency mannose 6-phosphate receptor or a low-efficiency endocytosis which may be independent of the mannose 6-phosphate receptor [13]. Thus the rate of uptake is dependent on whether the enzyme is phosphorylated (precursor) or dephosphorylated (mature form). Recently, it was reported that the mannose 6-phosphate receptor may also be present on the membrane of cultured muscle [11]. The apparent uptake constant reported here is compatible with the endocytosis of mature acid α-glucosidase since it is higher than that reported for the precursor forms of both acid α-glucosidase and other lysosomal enzymes by fibroblasts [11,13]. In the presence of 2 mM mannose 6-phosphate, uptake of acid α-glucosidase activity, from our preparation, was inhibited by approx. 20% (not shown). These data suggest that the enzyme preparation used here contained predominantly mature enzyme which may have been contaminated by small amounts of phosphorylated precursor [11].

The effect of acid α-glucosidase treatment on the glycogen concentration in cultured muscle from animals affected by glycogen type II is shown in fig.2. The acid α-glucosidase activity in homogenates of treated cultures was 10.9 ± 0.37 (5) nmol/h per mg protein, or about 10% of the

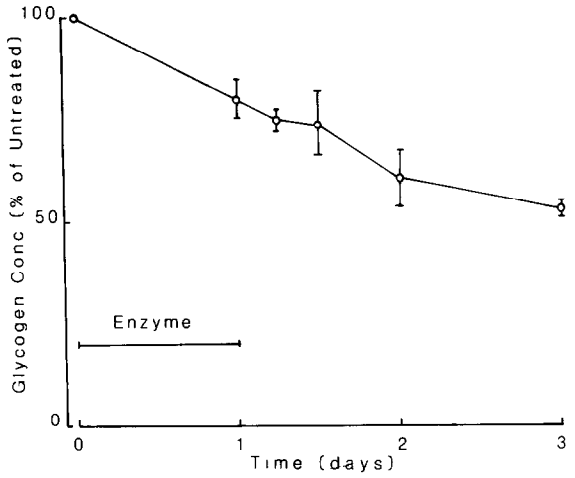

Fig.2. Glycogen concentration in cultured muscle treated with acid α-glucosidase. Day 6 cultures from affected animals were incubated with purified acid α-glucosidase (0.8 μmol/h per ml) in 2 ml uptake medium for 24 h as described in the text. The glycogen concentration in treated cultures is expressed as a percentage of the concentration in control cultures. Each point is the mean of 3–5 determinations from individual dishes.

activity in cultured muscle from non-affected animals [4]. In these cultures (fig.2), the concentration of glycogen was significantly lower ($p < 0.01$) than in untreated cultures by 24 h after the addition of the enzyme. By 48 h the glycogen concentration in treated cultures was 50.8 ± 6.4 (5) compared to 83.9 ± 14.3 (4) μg/mg protein in the untreated cultures, and was comparable to the concentration of glycogen in similar aged cultures from non-affected animals [4]. The concentration of glycogen in mature cultures from affected animals was approximately twice that in cultures from non-affected animals, and the glycogen appeared to be stored within lysosomes [4]. Results from electron microscopic studies suggest that the lysosomal glycogen is decreased after treatment with exogenous enzyme (Di Marco, unpublished). Our results thus show that purified acid α-glucosidase is taken up by cultured skeletal muscle from animals affected by glycogenosis type II. Moreover, intracellular levels of acid α-glucosidase activity equivalent to 10% of the activity in normal muscle cultures appear to be sufficient for the hydrolysis of the stored lysosomal glycogen. This is consistent with data on enzyme activity and

glycogen concentration in heterozygotes for glycogenosis type II and in patients affected by the adult onset form of the disease [2,3].

ACKNOWLEDGEMENTS

We wish to thank John Daly for skilful technical assistance. The work was supported by grants from the Australian Research Grant Scheme, Murdoch University and The Muscular Dystrophy Association of New York.

REFERENCES

[1] Howell, R.R. and Williams, J.C. (1983) in: Metabolic Basis of Inherited Diseases (Stanbury, J.B. et al. eds) 5th edn, pp.141–166, McGraw-Hill, New York.

[2] Di Mauro, S., Mehler, M., Arnold, S. and Miranda, A. (1977) in: Pathogenesis of Human Muscular Dystrophies (Rowland, L.P. ed.) pp.506–514, Excerpta Medica, Amsterdam.

[3] Howell, J.McC., Dorling, P.R. and Cook, R.D. (1983) Comp. Pathol. Bull. 15, 2–4.

[4] Di Marco, P.N., Howell, J.McC. and Dorling, P.R. (1984) Neuropathol. Appl. Neurobiol. 10, 379–395.

[5] Hug, G. and Schubert, W.K. (1967) J. Cell Biol. 35, C1–C6.

[6] De Barsy, T. and Van Hoof, F. (1974) in: Enzyme Therapy in Lysosomal Storage Diseases (Tager, J.M. et al. eds) pp.277–279, North-Holland, Amsterdam.

[7] De Barsy, T. (1976) Thesis, Université Catholique de Louvain, Oyez, Louvain.

[8] Williams, J.C., Chambers, J.P., Di Marco, P.N., Dorling, P.R. and Howell, J.McC. (1981) Pediatr. Res. 15, 571.

[9] Dorling, P.R., Howell, J.McC. and Gawthorne, J.M. (1981) Biochem. J. 198, 409–412.

[10] Reuser, A.J.J. and Kroos, M. (1982) FEBS Lett. 146, 361–364.

[11] Reuser, A.J.J., Kroos, M.A., Ponne, N.J., Wolterman, R.A., Loonen, M.C.B., Busch, H.F.M., Visser, M.J. and Bolhuis, P.A. (1984) Exp. Cell Res. 155, 178–189.

[12] Bruni, C.B., Auricchio, F. and Covelli, I. (1969) J. Biol. Chem. 244, 4735–4742.

[13] Ullrich, K., Mersmann, G., Weber, E. and Von Figura, K. (1978) Biochem. J. 170, 643–650.