Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 1047-1053

THE UPTAKE OF NATIVE AND DESIALYLATED GLUCOCEREBROSIDASE BY RAT HEPATOCYTES AND KUPFFER CELLS

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Received March 2, 1978

Summary

Human glucocerebrosidase is cleared from rat circulation after intravenous administration with a half-life of 21 minutes. The enzyme retains at least 85% of its activity following desialylation with neuraminidase and the half-life of the desialylated enzyme is 1.2 minutes. Asialo-orosomucoid inhibits the disappearance of the desialylated enzyme from the blood. Untreated enzyme is taken up by both Kupffer cells and hepatocytes while desialylated enzyme is cleared predominantly by hepatocytes and the uptake by Kupffer cells is reduced. These findings indicate that both Kupffer cells and hepatocytes take up a lysosomal enzyme and removal of sialic acid augments uptake by hepatocytes and decreases uptake by Kupffer cells.

Introduction

Intravenously administered glycoprotein lysosomal acid hydrolases are rapidly cleared from the circulation of the rat (1) and most of the infused enzymes can be recovered in the liver (2). The kinetics of the clearance are complex being made up of at least one "fast" component and one "slow" component. Most of the administered enzymes are cleared by the fast pathway and this process is saturable. The relative amount of enzyme cleared by the slow pathway varies with different preparations of enzyme and is always a small portion of the uptake (2). Ashwell and Morell have shown that the liver has specific receptor sites on hepatocytes which recognize galactose (mammalian) and N-acetylglucosamine (avian) terminals of glycoproteins and mediate their rapid clearance from the blood (3, 4). Oxidation by periodate destroys the rapid clearance of the acid hydrolases and permits only a slow uptake of these enzymes by the liver (5). Sly et al (6) have

0006-291X/78/0813-1047$01.00/0

suggested that Kupffer cells play the major role in the rapid uptake of lysosomal acid hyrolases from the circulation (7) and that mannose and N-acetylglucosamine terminal sugar residues can inhibit this Kupffer cell mediated uptake. Neufeld has illustrated the importance of sialic acid in the rapid uptake of acid hydrolases by fibroblasts (8). Furthermore, the rapid clearance of desialylated acid hydrolase has been reported (9). The deficiency of sialidase in I-cell disease may be responsible for the low cellular concentration of several lysosomal enzymes (10) since the excess sialic acid-containing enzymes (11) may be poorly taken up. These observations prompted us to investigate the relative importance of the hepatocyte and the Kupffer cell in mediating clearance of a native glycoprotein acid hydrolase and its desialylated form.

Methods and Materials

Infusion Enzymes: Highly purified human placental glucocerebrosidase was prepared by the method of Furbish et al (12) which was modified to utilize small-scale laboratory procedures (13). The method consisted of cholate extraction, ammonium sulfate fractionation, exhaustive dialysis, butanol extraction, ethanol fractionation, and hydrophobic chromatography of the placental extracts. Following the final chromatographic step, preparations of enzyme with specific activities of 8.0×10^5 to 1.6×10^6 units per mg protein were pooled and stored in 60% ethylene glycol containing 100 mM sodium citrate, pH 5.0, with 5 mM EDTA and 1 mM dithiothreitol. Prior to infusion or neuraminidase treatment, the ethylene glycol was removed by dialysis against 25 mM acetate, pH 5.0; approximately 35% of the activity was lost upon the removal of the ethylene glycol.

Asialo-glucocerebrosidase was obtained by incubating aliquots of the enzyme in 25 mM acetate, pH 5.0, with an excess of agarose-bound neuraminidase (Sigma product # N-5254) for up to 30 minutes at 37°. The agarose-neuraminidase was removed by centrifugation and the supernatant dialyzed against 25 mM acetate, pH 5.0. The neuraminidase incubation and subsequent dialysis usually resulted in an additional 15% loss of activity. Once prepared, the desialylated enzyme was stable for several days with no further loss in activity.

Enzyme Assays: Enzymic activities were determined as described previously (12) and are expressed as nanomoles glucocerebroside hydrolyzed per hour. Activities in cell preparations were obtained by assay of detergent-buffer extracts in which a known quantity of Kupffer cells or hepatocytes (usually 5×10^6 cells) was sonicated in 0.5 ml 15 mM phosphate, pH 6.0, containing 10 mg/ml sodium taurocholate and 2 mg/ml cutscum.

Enzyme Infusion: Male Osborne-Mendel rats (400 g) were anesthetized by injecting sodium pentobarbital intraperitoneally. The femoral vein and artery were cannulated for enzyme infusion and blood sampling. Enzyme infusions varied from 1.6×10^5 units for half-life studies and up to 1.0×10^6 units for liver uptake studies. Samples of blood were withdrawn

at intervals to verify completion of plasma disappearance before liver cells were isolated. In the competition studies, 5 mg of asialo-orosomucoid were administered intravenously before infusion of desialylated enzyme.

Isolation of Hepatocytes: Modifications of the method of Berry and Friend (14) for the preparation of isolated liver cells were as follows: (a) The apparatus and liver perfusion technique were as described by Hems, et al (15) as modified by Krebs et al (16). (b) Calcium-free Krebs-Henseleit buffer was used as the perfusate. (c) The concentration of collagenase was 0.03%; hyaluronidase was not added. (d) The gas phase was 95% O_2/5% CO_2. (e) A perfusion rate of 20-25 ml/min was used. After approximately 25 minutes, the perfusion was discontinued. The liver, 10-12 grams, was cut into small pieces and transferred to 100 ml Krebs-Henseleit solution, pH 7.4. The mixture was gently swirled for 2 minutes at 30°C. The cell suspension was filtered several times through nylon mesh 0.5 mm X 0.3 mm and was left for 5 minutes at 4°C to permit cells to sediment by gravity. The supernatant, which contained non-parenchymal cells was pipetted off leaving the hepatocytes in 8-10 ml of solution. 30 ml of fresh Waymouth's media 752/1 (17) (to which had been added bovine serum albumin to a final concentration of 1.3%) was added and the suspension gently mixed. Cells were counted by hemocytometer.

Isolation of Kupffer Cells: Modifications of the method described by Munthe-Kaas and Seglen (18) were as follows: (a) The initial cell suspension was prepared in Krebs-Henseleit solution. (b) A single solution of Metrizamide was used at a final concentration of 16% (w/v) (16 g Metrizamide, 0.24 g HEPES, 0.05 g KCl, 0.018 g $CaCl_2.2H_2O$, 5.5 ml 0.1 M NaOH and H_2O ad 100 ml), pH 7.6 at 37°C. Tubes containing 13 ml of this solution onto which was layered 25 ml portions of the supernatant obtained from the hepotocyte isolation were placed in a SW 27 Beckman rotor and centrifuged at 4°C for 45 min at 3000 X g. The purified non-parenchymal cells which included Kupffer cells were recovered by pipette at the Metrizamide interface. The cell suspension was washed with 50 ml Krebs-Henseleit buffer, pH 7.4 and centrifuged at 500 X g for 3 min. Cells were resuspended in 4 ml Waymouth's media 752/1 with 1.3% bovine serum albumin and counted by hemocytometer.

Results and Discussion

Figure 1 shows the clearance of 1.6 X 10^5 units each of neuraminidase-treated and untreated glucocerebrosidase from blood. The clearance of the treated enzyme was 17 times faster than that of native glucocerebrosidase. Incubation of the enzyme with less than an excess of neuraminidase gave half-lives with values between 21 and 1.2 minutes, but the lower figure could not be further decreased by adding additional neuraminidase or incubating for a longer time. A control of glucocerebrosidase incubated in the presence of agarose alone gave the same half-life as that of the untreated enzyme. Figure 1 also shows the clearance of asialo-enzyme in the presence of asialo-orosomucoid. The inhibition of clearance is typical of that observed for galactose-terminal glycoproteins (3). These observations suggest that the

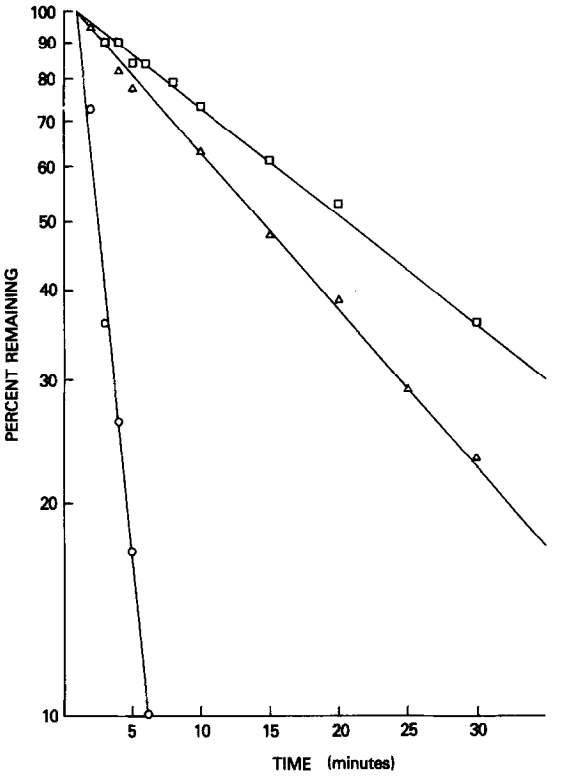

Figure 1. Clearance of glucocerebrosidase from the circulation of rats. Results are expressed as a percentage of the first post-infusion sample taken. □ , untreated enzyme ($t_{1/2}$ = 21 min.); O , asialo-enzyme ($t_{1/2}$ = 1.2 min.); Δ , asialo-enzyme + 5 mg asialo-orosomucoid ($t_{1/2}$ = 13.5 min). Each point is the average of three determinations.

asialo derivative of glucocerebrodisase has been formed. This is the first reported hepatic uptake study of a desialylated lysosomal enzyme.

The distribution of exogenous glucocerebrosidase in the liver following intravenous infusion is indicated in Table 1. After the infusion of either untreated and desialylated enzyme preparations, both hepatocyte and Kupffer cell populations showed an increase in enzymic activity over baseline levels. Kupffer cell assimilation was about three times greater for the untreated enzyme than for the asialo-enzyme, whereas uptake of the asialo-enzyme was distinctly favored by hepatocytes. For both intact and asialo preparations, the majority of enzyme was incorporated by hepatocytes. Because of some cell destruction that occurs

TABLE 1

Hepatic Uptake of Glucocerebrosidase

Experiment		Units Administered	Enzyme Activity units per 10^6 cells	
			Kupffer cells[a]	Hepatocytes[b]
1.	Glucocerebrosidase	1.0×10^6	69.0	281
		6.5×10^5	34.0	243
		1.0×10^6	39.7	240
	Average (SEM)		47.6 (± 10.9)	255 (± 13.2)
2.	Neuraminidase-treated Glucocerebrosidase	8.8×10^5	17.0	371
		7.0×10^5	15.8	268
		5.1×10^5	14.6	371
	Average (SEM)		15.8 (± 0.7)	337 (± 34.3)

[a] Baseline value = 6.6 (± 0.9) units/10^6 cells.

[b] Baseline value = 103.6 (± 6.4) units/10^6 cells.

during the isolation techniques, the total cellular uptake could not be quantitated.

The prompt disappearance of the asialo-enzyme from the circulation, inhibition of its clearance by asialo-orosomucoid, and its relatively greater assimilation by hepatocytes suggests that the asialo-enzyme behaves like other asialo-glycoproteins in mammals which are specifically taken up by hepatocytes as a direct consequence of the specific interaction of exposed galactosyl residues with the hepatic binding protein described by Ashwell and Morrell (3). The extensive involvement of hepatocytes in the incorporation of both untreated and desialyliated enzyme is suprising in light of the clearance of the lysosomal enzyme β-glucuronidase which has been reported as Kupffer cell mediated with N-acetylglucosamine and mannose terminals as recognition factors (6).

Our observations would suggest the importance of a specific membrane asialo

receptor in the rat hepatocyte in the uptake of the desialylated lysosomal enzyme glucocerebrosidase. In vitro studies of isolated rat hepatocytes show specific binding of galactose, N-acetylglucosamine, and mannose terminal glycoproteins, whereas Kupffer cells have only N-acetylglucosamine and mannose receptors (19). Other studies have revealed that the clearance of several native lysosomal enzymes are not inhibited by asialo-orosomucoid (20) indicating the absence of galactose terminals on these glycoproteins. The removal of sialic acid from glucocerebrosidase by neuraminidase apparently exposes galactose moieties and permits uptake via the specific asialoglycoprotein hepatic binding protein located in the surface membrane of the hepatic parenchymal cell. The presence of a sialidase in the circulation or on the membranes of cells could play an important role in the series of steps proceeding from secretion to recapture of lysosomal acid hydrolases. The relatively greater number and mass of hepatocytes (21) may account for the predominant location of lysosomal enzymes in these cells (22) if such a sialidase operates in the presence of the specific hepatic binding protein for asialoglycoproteins.

Acknowledgement

We thank Dr. Gilbert Ashwell for the kind contribution of asialo-orosomucoid.

The excellent technical assistance of Wendy Fredericks is gratefully acknowledged.

References

1. Stahl, P., Rodman, J.S., and Schlesinger, P. (1976) Arch. Biochem. Biophys. 177, 594-605.
2. Schlesinger, P., Rodman, J.S., Frey, M., Lang, S., and Stahl, P. (1976) Arch. Biochem. Biophys. 177, 606-614.
3. Ashwell, G., and Morrell, A. (1974) Adv. in Enzymol. 41, 99,128.
4. Lunney, J., and Ashwell, G. (1976) Proc Nat. Acad. Sci. USA, 73, 341-343.
5. Achord, D., Brot, F., Gonzalez-Noriega, A., Sly, W., and Stahl, P. (1977) Pediat. Res. 11, 816-822.
6. Stahl, P., Six, H., Rodman, J.S., Schlesinger, P., Tulsiani, D.R.P., and Touster, O. (1976) Proc. Natl. Acad. Sci. USA, 73, 4045-4049.
7. Achord, D., Brot, F., and Sly, W. (1977) Biochem. Biophys. Res. Commun., 77, 409-415.

8. Neufeld, E., Sardo, G.N., Garvin, A.J., and Rome, L.H. (1977) J. Supramolec. Struct. 6, 95-101.
9. Bearpark, T., and Stirling, J.L. (1977) Biochem. J. 168, 435-439.
10. Vladutiu, G.D., and Rattazzi, M.C. (1975) Biochem. Biophys. Res. Commun., 67, 956-964.
11. Thomas, G.H., Tiller, G.E., Reynolds, L.W., Miller, C.S., and Boce, J.W. (1976) Biochem. Biophys. Res. Commun. 71, 188-195.
12. Furbish, F.S., Blair, H.E., Shiloach, J., Pentchev, P.G., and Brady, R.O. (1977) Proc. Natl. Acad Sci. USA, 74, 3560-3563.
13. Furbish, F.S. Manuscript in preparation.
14. Berry, M.N., and Friend, D.S. (1969) J. Cell Biol. 43, 506-520.
15. Hems, R., Ross, B.D., Berry, M.N., and Krebs, H.A. (1966) Biochem. J. 101, 284-292.
16. Krebs, H.A., Cornell, N.W., Lund, P., and Hems, R. (1973) in Regulation of Hepatic Metabolism, Lundquist, F. and Tygstrup, N. (eds), pp. 726-750, Academic Press, New York.
17. Waymouth, C. (1959) J. Nat. Cancer Inst. 22, 1003-1017.
18. Munthe-Kaas, A.C., and Seglen, P.O. (1974) FEBS Letters 43, 252-256.
19. Steer, C.J., and Hickman, J. Manuscript in preparation.
20. Stahl, P., Schlesinger, P., Rodman, J.S., and Doebber, T. (1976) Nature 264, 86-88.
21. Blouin, A., Bolender, R.P., and Weibel, E.R. (1977) J. Cell Biol. 72, 441-455.
22. Barranger, J.A., Furbish, F.S., and Pentchev, P.G. Manuscript in preparation.