THE UPTAKE OF AGALACTO-GLUCOCEREBROSIDASE BY RAT HEPATOCYTES AND KUPFFER CELLS

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1. Introduction

The rapid clearance of intravenously infused lysosomal enzymes appears to be a general phenomenon in the rat. These enzymes are predominantly glycoproteins and specific recognition sites on their carbohydrate moieties are apparently involved in mediating the rapid clearance process [1]. Presumably, subtle structural differences in the nature of the oligosaccharide fragment of these enzymes are capable of greatly modifying or even eliminating the binding reaction and subsequent clearance from the blood [2].

The isolation and characterization of an hepatic membrane protein which specifically recognizes and binds the terminal galactosyl residues of glycoproteins has been described [3,4]. The binding protein appears to be responsible for the rapid and selective hepatic clearance of desialylated glycoproteins from the circulation [2]. An avian hepatic binding protein which recognizes and binds specifically to serum glycoproteins bearing terminal non-reducing *N*-acetylglucosamine residues has also been identified and isolated [5,6]. However, no such hepatic receptor has been isolated from mammals. The rapid plasma clearance of lysosomal glycosidases seems to be mediated by liver plasma membrane binding sites or receptors with subsequent endocytosis [7]. Evidence suggests that these lysosomal enzymes localize in Kupffer cells and that a substantial part of the clearance is mediated by a mannose recognition system. Both mannans and *N*-acetylglucosamine terminated glycoproteins can inhibit uptake of lysosomal enzymes by the liver [8].

In order to elucidate the mechanism of hepatic uptake of lysosomal acid hydrolases, the clearance of intravenously infused native and desialylated glucocerebrosidase by rat hepatocytes and Kupffer cells has been studied [9]. Their findings indicated that intact glucocerebrosidase is cleared by both hepatocytes and Kupffer cells and that removal of sialic acid from the enzyme markedly increased its uptake by hepatocytes with a corresponding decrease in uptake by Kupffer cells after intravenous infusion. Furthermore, agalacto-orosomucoid was found [1,7] to block the clearance of a variety of rat lysosomal enzymes which are normally cleared rapidly following infusion. These findings led them to attribute clearance of the lysosomal hydrolases to the system described for agalacto-orosomucoid and to suggest that the terminal *N*-acetylglucosamine was part of the recognition site on the enzyme which mediated clearance.

These results and proposals prompted us to investigate the importance of hepatocytes and Kupffer cells in mediating clearance of glucocerebrosidase from which both sialic acid and galactose had been enzymatically cleaved.

2. Materials and methods

2.1. *Infusion enzymes*

The preparation of human placental glucocerebrosidase and its desialylated form have been described previously [9,10]. The agalacto enzyme was prepared by reacting purified β-galactosidase [11] with asialoglucocerebrosidase. In a typical preparation, 10–20 μl 5.5 μmol/min/ml β-galactosidase were incubated with

$0.6-1.5 \times 10^6$ units asialoglucocerebrosidase (390–900 μg protein) in 50 mM citrate–phosphate buffer, pH 6.5. Because of the relatively small amount of β-galactosidase with respect to that of the glucocerebrosidase, the two enzymes were not separated prior to infusion. Incubation with β-galactosidase caused no loss of glucocerebrosidase activity.

2.2. *Enzyme assays*

Enzyme activities were determined as in [10] and are expressed as nmol glucocerebroside hydrolyzed/h. Activities in cell preparation were obtained by assay of detergent–buffer extracts in which a known quantity of hepatocytes or Kupffer cells (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.

2.3. *Enzyme infusion*

Male Osborne-Mendel rats were anesthetized by injecting sodium pentobarbital intraperitoneally. The femoral vein and artery were cannulated for enzyme infusion and blood sampling, respectively. Agalacto-enzyme, ~600 000 units, were infused to measure plasma half-life, while 650 000 units were infused to study liver uptake. To determine whether the clearance of agalacto-glucocerebrosidase could be inhibited by another agalacto-glycoprotein, agalacto-fetuin, 5 mg, was administered intravenously 2 min before infusion of agalacto-glucocerebrosidase.

2.4. *Isolation of hepatocytes*

Modifications of the method of Berry and Friend for the preparation of isolated liver cells were as in [9]. In short, the apparatus and liver perfusion technique were as in [12] modified [13]. Calcium-free Krebs-Henseleit buffer was used as the perfusate. No hyaluronidase was added. No adjustment of pH was needed with a gas phase of 95% O_2/5% CO_2. A perfusion rate of 20–25 ml/min was used.

Total recovery ranged from $25-50 \times 10^7$ hepatocytes/rat liver perfused. From 80–90% of the cells excluded Trypan blue. Contamination by non-parenchymal cells was < 1%.

2.5. *Isolation of Kupffer cells*

The isolation technique was essentially as in [9]. The initial cell suspension was prepared in Krebs-Henseleit solution. A single solution of Metrizamide was used at final conc. 16% (w/v). The purified non-parenchymal cells which included Kupffer cells were recovered by pipette from the Metrizamide–buffer interface. Kupffer cells present in the suspension were identified by their ability to phagocytize latex and carbon particles as well as their ability to bind to immunoglobulin (IgG) coated sheep red blood cells [14,15]. Using these parameters, ~25% of the cells were identified as Kupffer cells and the remainder as endothelial cells. Total recovery of sinusoidal cells ranged from $20-35 \times 10^6$ cells/rat liver perfused. In excess of 95% of the cells excluded Trypan blue; contamination with hepatocytes was < 0.5%.

3. Results and discussion

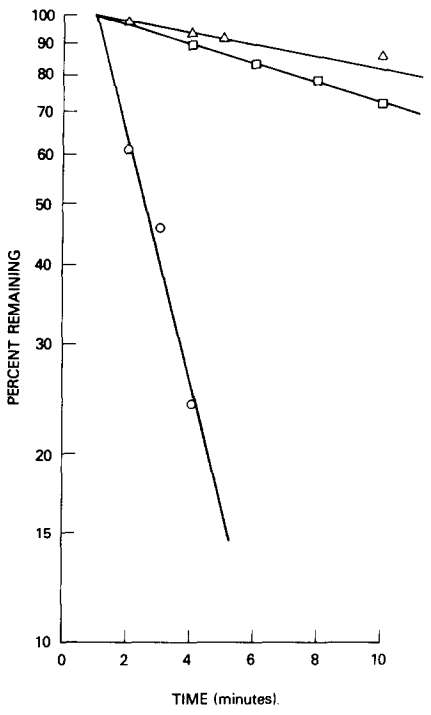
The distribution of intact and enzymatically-modified glucocerebrosidase following intravenous infusion is shown in table 1. Infusion of untreated enzyme resulted in ~7-fold increase in enzyme content by Kupffer cells and ~2.5-fold increase in hepatocyte content over baseline [9]. Considering that hepatocytes constitute 78% parenchymal volume while sinusoidal lining cells constitute only 6% in the rat [16], it is evident that the majority of the infused enzyme is taken up by hepatocytes. Infusion of asialoglucocerebrosidase [9] resulted in an appreciably larger proportion of enzyme taken up by hepatocytes and a correspondingly smaller proportion being taken up by Kupffer cells. The present study shows that after further enzymatic modification of the enzyme by treatment with β-galactosidase, intravenous infusion of the agalacto-enzyme also results in an increased amount of enzymatic activity in hepatocytes. However, in contrast to the results obtained with the asialo-enzyme, there is also a marked increase in the uptake of the agalacto-enzyme by Kupffer cells, the enzymatic content of these cells being 25-times greater than that of corresponding cells from control animals.

Figure 1 shows plasma disappearance curves of intact and agalacto-glucocerebrosidase. The clearance of the modified enzyme was 14-times faster than that of the native glucocerebrosidase. In [9], the clearance of asialoglucocerebrosidase was also found to be appreciably faster than that of the intact enzyme. Figure 1 also shows that the accelerated clearance of

Table 1
Hepatic uptake of glucocerebrosidase

	Units admin.	Enzyme activity (units/10^6 cells)	
		Kupffer cells[a]	Hepatocytes
Baseline gluco-cerebrosidase (SEM)	0	6.6 (± 0.9)	103.6 (± 6.4)
Intact gluco-cerebrosidase	1.0×10^6	69.0	281
	1.0×10^6	39.7	240
	6.5×10^5	34.0	281
Average (SEM)		47.6 (± 10.9)	255 (± 13.2)
β-Galactosidase and neuraminidase-treated glucocerebrosidase	6.5×10^5	191	432
	6.5×10^5	125	378
	6.3×10^5	193	328
Average (SEM)		169.7 (± 22.3)	379.3 (± 30.0)

[a] No distinction between Kupffer cells and other sinusoidal lining cells was made in determination of cell number



agalacto-glucocerebrosidase is completely blocked in the presence of excessive agalacto-fetuin. This inhibition of clearance tends to confirm that the enzymatically-modified glucocerebrosidase used in this study was the agalacto-derivative. Quantitative carbohydrate analyses are currently in progress to determine the exact amounts of sialic acid and galactose removed from glucocerebrosidase by the enzymatic procedures used in this study.

The recognition system for clearance of agalacto-glucocerebrosidase cannot at this time be precisely defined. A substantial part of the clearance of intact lysosomal enzyme from rat plasma may be mediated by a mannose recognition system [1,8]. A mannan-binding protein in rabbit liver has been isolated and characterized [17]. *N*-Acetylglucosamine-terminated

Fig.1. Clearance of glucocerebrosidase from the circulation of rats. Results are expressed as a percentage of the first post-infusional sample taken. Each point represents the mean value from 3 expts.
(□) Untreated enzyme ($T_{1/2}$ 21 min).
(○) Agalacto-enzyme ($T_{1/2}$ 1.5 min).
(△) Agalacto-enzyme + 5 mg agalacto-fetuin ($T_{1/2}$ 32 min).

glycoproteins were in fact potent inhibitors of mannan binding to this protein. The mannan-binding protein was proposed to be the principle mediator of hepatic uptake of *N*-acetylglucosamine-terminated glycoproteins [17]. However, the exact role of the mannan binding protein in the recognition and uptake of lysosomal enzymes remains to be established.

Several explanations are possible for the observed greater uptake of agalacto-glucocerebrosidase by Kupffer cells than desialylated glucocerebrosidase. Kupffer cells may have surface receptor(s) which specifically recognize *N*-acetylglucosamine-terminated glycoproteins. Alternatively, there may be further enzymatic modification of the enzyme, either by Kupffer cell or in the circulation, which renders it more susceptible to uptake by Kupffer cells. Unpublished results from this laboratory suggest the former explanation.

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