Endocytosis of Lysosomal Enzymes by Non-Parenchymal Rat Liver Cells

COMPARATIVE STUDY OF LYOSOMAL-ENZYME UPTAKE BY HEPATOCYTES AND NON-PARENCHYMAL LIVER CELLS

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Cultured non-parenchymal rat liver cells internalize human urine α -N-acetylglucosaminidase, human skin β -N-acetylglucosaminidase and pig kidney α -mannosidase. Different heat-stabilities of endocytosed and endogenous α -mannosidase activity provided indirect evidence that the increase in intracellular activity resulted from uptake. The high efficiency and the saturation kinetics of uptake indicated that these enzymes become internalized by adsorptive endocytosis. Competition experiments with glycoproteins bearing known carbohydrates at their non-reducing terminals, with mannans, methyl glycosides and monosaccharides, established that the uptake of these three lysosomal enzymes is mediated by the binding to cell-surface receptors that recognize mannose and N-acetylglucosamine residues. The decreased uptake after treatment of these enzymes with either β -N-acetylglucosaminidase or α -mannosidase was in accordance with the results of the inhibition experiments. Removal of oligosaccharides of the high-mannose type by treatment with endoglucosaminidase H inhibited uptake almost completely, suggesting that the sugars recognized by cell-surface receptors of non-parenchymal liver cells are located in the outer core of these oligosaccharides. A comparison of the uptake of these three lysosomal enzymes by parenchymal and non-parenchymal rat liver cells indicates that infused α -N-acetylglucosaminidase is taken up preferentially by hepatocytes, whereas α -mannosidase and β -N-acetylglucosaminidase are localized predominantly in non-parenchymal rat liver cells.

Intravenously infused lysosomal enzymes are rapidly cleared from the plasma by the recognition of their carbohydrate side chains (Stahl *et al.*, 1976a,b; Achord *et al.*, 1977a, 1978). Liver appears to be the organ chiefly responsible for the clearance of lysosomal enzymes (Thorpe *et al.*, 1974; Marinkovic *et al.*, 1976; Achord *et al.*, 1977a; Fiddler & Desnick, 1977; Schlesinger *et al.*, 1977). Liver cells expose at their cell surface receptors that recognize terminal galactose and N-acetylgalactosamine/glucose residues or N-acetylglucosamine and mannose/glucose residues on glycoproteins (for review see Neufeld & Ashwell, 1978). Binding of glycoproteins to these receptors appears to be followed by uptake and delivery of the ligand into the lysosomal apparatus. Recently the recognition of lysosomal enzymes by hepatocytes via galactose (Furbish *et al.*, 1978; Ullrich *et al.*, 1979) and/or mannose 6-phosphate residues (Ullrich *et al.*, 1978b, 1979), and by sinusoidal lining cells of liver via N-acetylglucosamine and mannose residues (Achord *et al.*, 1978), has been reported. The present study is concerned with the characterization of the carbohydrates involved in the recognition of three lysosomal enzymes by isolated non-parenchymal rat liver cells and a

comparison of the uptake of these enzymes by isolated parenchymal and non-parenchymal liver cells.

Experimental

Material

Mannose 6-phosphate and sugars were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Boehringer Mannheim (Mannheim, Germany). *p*-Nitrophenyl 2-acetamido-2-deoxy- α -D- and β -D-glucopyranoside and *p*-nitrophenyl α -D- and β -D-mannopyranoside were purchased from Koch-Light (Colnbrook, Bucks., U.K.); endoglucosaminidase H was from Seikagaku (Tokyo, Japan); collagenase I (EC 3.4.24.3), β -N-acetylglucosaminidase from jack bean (EC 3.2.1.30), horseradish peroxidase and yeast mannan were from Sigma. Orosomucoid was a gift from the American Red Cross Research Laboratory (Bethesda, MD, U.S.A.). *p*-Aminophenyl 2-acetamido-2-deoxy-1-thio- β -D-glucopyranoside was obtained from Bachem Chemicals (Torrance, CA, U.S.A.); Sepharose 4B, CH-Sepharose 4B and Percoll were from Pharmacia (Uppsala, Sweden).

Asialo derivatives of orosomucoid were prepared by acid hydrolysis (Stockert *et al.*, 1976), and agalacto-orosomucoid was prepared as described by Spiro (1966). Mannan fractions I and VIII were obtained by separation according to charge as described by Ullrich *et al.* (1978b), fraction I containing 16mmol and fraction VIII 216mmol of phosphate/mg of mannan. Female Wistar rats were provided by Hannoversche Versuchstieranstalt (Hannover, Germany).

Liver perfusion

Perfusion and isolation of hepatocytes were done as described by Ullrich *et al.* (1979) by the procedure of Seglen (1976). The cell suspension obtained after liver perfusion was centrifuged at 65g for 3min. The supernatant containing the non-parenchymal cells was centrifuged twice at 65g for 3min in ice-cold Eagle's minimal essential medium (Eagle, 1959) containing 10% foetal calf serum (LS-Labor Service, München, Germany). The final supernatant was centrifuged for 5min at 850g. Of the pelleted non-parenchymal cells 20×10^6 cells were layered on a 22ml Percoll gradient in sucrose with a starting density of 1.03g/ml, and centrifuged as described for hepatocytes (Ullrich *et al.*, 1979). The non-parenchymal cells banded near the top, whereas the remaining hepatocytes assembled near the bottom of the gradient. The final preparation of non-parenchymal cells contained less than 1% non-viable cells as judged by exclusion of 0.1% (w/v) Trypan Blue.

Cell culture

Hepatocytes were cultured as described by Ullrich *et al.* (1979). Cultures were free of macrophages, as demonstrated by the lack of phagocytosis of (a) ink (Günther Wagner, Hannover, Germany) and (b) fluorescein isothiocyanate-conjugated immunoglobulin G (Berhringwerke, Marburg, Germany). Non-parenchymal cells (1.2×10^6 – 1.5×10^6) were plated on 35mm-diameter Falcon plastic dishes in Eagle's medium containing 20% foetal calf serum. The cultures were completely free of hepatocytes. The amount of macrophages in this cell fraction was 50–60% as estimated from phagocytosis of ink and 40–70% as judged from uptake of fluorescein isothiocyanate-conjugated immunoglobulin G.

Enzyme preparation

Human urine α -N-acetylglucosaminidase (EC 3.2.1.50) was purified to homogeneity as described by von Figura (1977a) to a specific activity of 2.0units/mg of protein. Pig kidney α -mannosidase

(EC 3.2.1.24) was obtained after purification step 5 of Mersmann & Buddecke (1977) with a specific activity of 3units/mg of protein. β -N-Acetylglucosaminidase from human skin fibroblasts was solubilized by ten freeze-thaw cycles. The supernatant was applied to a column of CH-Sepharose 4B substituted with *p*-aminophenyl 2-acetamido-2-deoxy-1-thio- β -D-glucopyranoside, equilibrated and eluted under conditions as described by Glasgow *et al.* (1977). The final preparation had a specific activity of 80units/mg of protein. A unit of enzyme activity is that amount of enzyme catalysing the reaction of 1 μ mol of substrate/min. Protein was determined as described by Lowry *et al.* (1951).

Endocytosis of lysosomal enzymes

Non-attached cells were removed by suction 6h after plating. Endocytosis was determined exactly as described for hepatocytes (Ullrich *et al.*, 1979), except that Eagle's minimal essential medium containing 20% foetal calf serum was used for non-parenchymal cells. Inhibitors were added in isotonic solution (0.9% NaCl) in a volume of up to 20% of total volume. Unless otherwise stated α -N-acetylglucosaminidase concentration was 2munits/ml. Standard concentration of α -mannosidase and β -N-acetylglucosaminidase in endocytosis experiments with non-parenchymal cells was 35munits/ml and 60munits/ml, respectively, whereas uptake by hepatocytes was measured in the presence of 320munits/ml and 1unit/ml, respectively. The period of endocytosis was 16h. Cells were harvested with trypsin; after ten cycles of freezing and thawing, protein (Kaltwasser *et al.*, 1965) and activities of α -N-acetylglucosaminidase, β -N-acetylglucosaminidase and α -mannosidase were assayed as described by Ullrich *et al.* (1978a). Uptake of lysosomal enzymes was calculated from the difference in enzyme activities between cells that had been incubated in the presence of enzyme and controls.

Treatment of lysosomal enzymes with glycosidases

Digestion of α -N-acetylglucosaminidase and α -mannosidase was done with 200munits of endoglucosaminidase H/ml for 6h at 37°C in 80mM-sodium phosphate (pH 5.5)/130mM-NaCl/0.01% bovine serum albumin in a final volume of 0.07–0.36ml.

Treatment of pig kidney α -mannosidase with β -N-acetylglucosaminidase from *Diplococcus pneumoniae* (final concentration 1.5units/ml) and with β -N-acetylglucosaminidase from jack bean (final concentration 2units/ml) and treatment of fibroblast β -N-acetylglucosaminidase with pig kidney α -mannosidase (final concentration 1unit/ml) were as reported by von Figura (1977b). Before endocytosis

α -mannosidase was separated from the β -*N*-acetylglucosaminidase by passage over the affinity column for β -*N*-acetylglucosaminidase as described above, and all enzyme preparations were dialysed overnight against 10mM-sodium phosphate, pH6.0, containing 0.15M-NaCl.

Results

Endocytosis of lysosomal enzymes

Primary cultures of non-parenchymal rat liver cells internalized α -*N*-acetylglucosaminidase from human urine, β -*N*-acetylglucosaminidase from human skin fibroblasts and α -mannosidase from pig kidney (Table 1). After incubation for 16h in the presence of these enzymes, the intracellular activities increased 8–55-fold compared with controls. For α -mannosidase it was shown that the increase of intracellular activity resulted from uptake. Whereas α -mannosidase of non-parenchymal rat liver cells is stable at 70°C, the α -mannosidase of cells incubated in the presence of pig kidney α -mannosidase showed a heat-lability very similar to that of pig kidney α -mannosidase (Fig. 1). The uptake of α -mannosidase was linear with time for up to 18h (Fig. 2). The kinetics of uptake were studied in detail for α -*N*-acetylglucosaminidase. With increasing concentrations of this enzyme in the medium, saturation of uptake was observed (Fig. 3). Half-maximal uptake was reached at 5 munits/ml of medium (K_{uptake} 8.2 nM-enzyme). At saturation a single non-parenchymal liver cell may internalize up to 43×10^3 α -*N*-acetylglucosaminidase molecules/h.

Inhibition of lysosomal-enzyme uptake by glycoproteins, mannans and sugars

Glycoproteins with terminal *N*-acetylglucosamine residues (agalacto-orosomucoid) or mannose residues (horseradish peroxidase) were found to inhibit the

uptake of the three lysosomal enzymes tested (Table 2). A phosphate-rich mannan (mannan VIII) was a less effective inhibitor than a phosphate-poor mannan (mannan I). Compounds known to interact with cell-surface receptors specific for galactose (asialo-orosomucoid) or for mannose 6-phosphate had no effect on lysosomal-enzyme uptake.

The effect of monosaccharides at 50mM concentrations on α -*N*-acetylglucosaminidase uptake was in accordance with the results obtained with glycoproteins and mannans. In the presence of *N*-acetylglucosamine, mannose and glucose, endocytosis was decreased to 46, 13 and 73% of that of controls

Fig. 1. Thermal stability of α -mannosidase
Pig kidney α -mannosidase (○), homogenate of non-parenchymal liver cells (□) and homogenate of non-parenchymal liver cells which had been incubated for 18h in the presence of α -mannosidase (●) were suspended in 20mM-sodium phosphate, pH 6.0, containing 0.15M-NaCl. After heating for up to 20min at 70°C the α -mannosidase activity was determined and expressed as percentage of initial activity.

Table 1. Uptake of α -*N*-acetylglucosaminidase, β -*N*-acetylglucosaminidase and α -mannosidase by cultured non-parenchymal rat liver cells

The cells were incubated for 16h in the presence or absence of the enzymes. All values are the means of duplicates. In parentheses is given the range observed in four different control experiments of non-parenchymal liver cells. Clearance is calculated from the difference between cells incubated with enzyme and controls. The clearance expresses that amount of medium cleared from enzyme/h per mg of cell protein (Lagunoff *et al.*, 1973).

Enzyme	Concn. in medium (munits/ml)	Intracellular activity (munits/mg of cell protein)	Clearance (μ l/h per mg of cell protein)
α - <i>N</i> -Acetylglucosaminidase	2	3.3 0.38 (0.20–0.55)	88
β - <i>N</i> -Acetylglucosaminidase	60	119 15.2 (12.1–18.2)	144
α -Mannosidase	35	122 2.2 (1.0–3.8)	214

respectively, whereas galactose had no effect. The methyl α -D-glycosides of mannose and glucose had a similar inhibitory potency to that of the corresponding monosaccharides (Table 2).

Treatment of lysosomal enzymes with glycosidases

α -Mannosidase is known to contain almost exclusively mannose and *N*-acetylglucosamine residues (Okumura & Yamashina, 1973; Mersmann *et al.*, 1978). Exhaustive digestion of α -mannosidase

with β -*N*-acetylglucosaminidase from either jack bean or *Diplococcus pneumoniae* (both free of β -mannosidase) abolished uptake of α -mannosidase completely (to less than 1% of that of controls). These results indicate that after removal of the β -*N*-acetylglucosamine residues the α -mannosidase removes all of its own α -mannosyl residues that could interact with the cell-surface residues.

Fig. 2. Uptake of α -mannosidase
Non-parenchymal liver cells were incubated for up to 18h in the presence of 40munits of pig kidney α -mannosidase/ml of medium. Vertical bars indicate the ranges of duplicates of the intracellular α -mannosidase activity. Controls had a basic α -mannosidase activity of 1.0munits/mg of cell protein.

Fig. 3. Uptake of α -*N*-acetylglucosaminidase as a function of enzyme concentration
Non-parenchymal liver cells were incubated for 16h in the presence of up to 16munits of α -*N*-acetylglucosaminidase/ml of medium. Uptake was calculated from the difference of intracellular enzyme activities between cells incubated in the presence of enzyme and controls.

Table 2. Inhibition by glycoproteins, mannans and methyl glycosides of lysosomal-enzyme uptake
For details see the text.

Addition	Concn.	Uptake (% of control)		
		α - <i>N</i> -Acetylglucosaminidase	β - <i>N</i> -Acetylglucosaminidase	α -Mannosidase
Orosomucoid	0.1 mg/ml	86	91	78
Asialo-orosomucoid	0.1 mg/ml	119	92	104
Agalacto-orosomucoid	0.1 mg/ml	30	13	13
Horseradish peroxidase	0.1 mg/ml	27	8	6
Mannan I	10 μ g/ml	10	<2*	19
Mannan VIII	10 μ g/ml	50	<2*	34
Mannose 6-phosphate	0.5 mM	103	112	106
Methyl α -D-mannoside	50mM	12	6	22
Methyl α -D-glucoside	50mM	56	79	38

* Concentrations of mannans I and VIII were 0.1 mg/ml of medium.

Treatment of fibroblast β -*N*-acetylglucosaminidase with pig kidney α -mannosidase decreased uptake to 33% of that of control. Since horseradish peroxidase and mannans produced a much higher inhibition, these results indicate that the removal of the α -mannosyl residues interacting with the receptors was incomplete. Endoglucosaminidase H splits the dichitobiosyl linkage of oligosaccharides of the high-mannose type (Tarentino & Maley, 1974; Tarentino *et al.*, 1974), leaving an *N*-acetylglucosamine residue *N*-glycosidically linked to an asparagine residue of the protein core. Oligosaccharides of the high-mannose type consist of mannose and *N*-acetylglucosamine (for review see Kornfeld & Kornfeld, 1976). Treatment of α -*N*-acetylglucosaminidase and α -mannosidase with endoglucosaminidase H decreases uptake to 5 and 14% of that of controls (Table 3), indicating that *N*-acetylglucosamine residues *N*-glycosidically linked to the protein core are not or only poorly recognized by cell-surface receptors recognizing mannose and *N*-acetylglucosamine residues (Achord *et al.*, 1977b, 1978; Stahl *et al.*, 1978).

Comparison of lysosomal-enzyme uptake by parenchymal rat liver cells

The clearance of α -*N*-acetylglucosaminidase, β -*N*-acetylglucosaminidase and α -mannosidase determined in isolated hepatocytes was 8–150 times lower than that observed in non-parenchymal cells (Table 4). It should be noted that for determination of clearance in hepatocytes 17- and 9-fold higher

concentrations of β -*N*-acetylglucosaminidase and α -mannosidase were used than those used in experiments with non-parenchymal cells.

The direct comparison of the clearance values may mislead if the situation *in vivo* is considered, since on a weight basis hepatocytes exceed non-parenchymal cells in the tissue by a factor of about 26 (Munthe-Kaas *et al.*, 1976). A calculation of the relative uptake as expected for a circulating enzyme shows that 3 times as much α -*N*-acetylglucosaminidase will localize in parenchymal as in non-parenchymal cells, whereas twice as much β -*N*-acetylglucosaminidase and almost 6 times as much α -mannosidase will localize in non-parenchymal as in parenchymal cells (Table 4).

Discussion

Isolated rat alveolar macrophages possess cell-surface receptors specific for terminal mannose, *N*-acetylglucosamine and glucose residues on lysosomal enzymes (Stahl *et al.*, 1978). Previous studies *in vivo* had indicated the presence of a receptor system in liver recognizing mannose and *N*-acetylglucosamine residues that is distinct from the well-characterized galactose/*N*-acetylgalactosamine/glucose-specific receptor on hepatocytes (for review see Neufeld & Ashwell, 1978). Kawasaki *et al.* (1978) reported the isolation and characterization of a mannan-binding protein from rabbit liver, which had the binding characteristics of the postulated mannose/*N*-acetylglucosamine receptor. Evidence for the localization of a mannose/*N*-acetylgluco-

Table 3. Effect of endoglucosaminidase H treatment on uptake of lysosomal enzymes

For details see the text. Addition of endoglucosaminidase H to the medium of cells had no effect on enzyme uptake.

Lysosomal enzyme (munits/ml of medium)	Endoglucosaminidase H	Clearance	
		(μ l/h per mg of cell protein)	(% of control)
α - <i>N</i> -Acetylglucosaminidase (1.0)	—	121	
	+	5.8	5
α -Mannosidase (30)	—	138	
	+	18	14

Table 4. Comparison of lysosomal-enzyme uptake by parenchymal and non-parenchymal rat liver cells

To calculate relative uptake the assumption was made that circulating lysosomal enzymes are available for hepatocytes and non-parenchymal cells under conditions *in vivo* to a similar extent. The weight distribution of parenchymal and non-parenchymal cells in liver was taken as 26:1 (Munthe-Kaas *et al.*, 1976).

Enzyme	Clearance (μ l/h per mg of cell protein) by		Relative uptake (% of total uptake by liver) into	
	Hepatocytes	Non-parenchymal cells	Hepatocytes	Non-parenchymal cells
α - <i>N</i> -Acetylglucosaminidase	10	88	75	25
β - <i>N</i> -Acetylglucosaminidase	2.9	144	34	66
α -Mannosidase	1.4	214	15	85

amine-recognizing receptor system involved in the clearance of human placenta β -glucuronidase on non-parenchymal rat liver cells was provided by Achord *et al.* (1978), using immunohistochemical techniques.

The present results strongly suggest that non-parenchymal liver cells internalize lysosomal enzymes by a receptor-mediated endocytosis. This is indicated by the saturation kinetics of uptake and the inhibition of endocytosis by a wide variety of compounds that have in common the exposure of terminal mannose or *N*-acetylglucosamine residues. The affinity of α -*N*-acetylglucosaminidase to the cell-surface receptors is similar to that previously reported for this enzyme to receptors present on human skin fibroblasts (von Figura, 1977b; Ullrich *et al.*, 1978a) and on rat hepatocytes (Ullrich *et al.*, 1979). However, the maximal velocity with which a non-parenchymal liver cell may internalize α -*N*-acetylglucosaminidase is less than 25% and 5% of that of rat hepatocytes or human skin fibroblasts respectively.

The effect of the various glycoproteins, mannans and sugars on uptake of the three lysosomal enzymes tested and the loss of uptake after treating the enzymes with glycosidases removing terminal *N*-acetylglucosamine and mannose residues suggest that non-parenchymal liver cells recognize these enzymes via receptors specific for mannose and *N*-acetylglucosamine residues. The loss of uptake after treatment with endoglucosaminidase H indicates that *N*-acetylglucosamine residues *N*-glycosidically linked to asparagine residues of the lysosomal-enzyme protein core are not or only poorly recognized by the mannose/*N*-acetylglucosamine-specific receptors.

This receptor system is clearly differentiated from the galactose/*N*-acetylgalactosamine-specific receptors by which hepatocytes internalize the same α -*N*-acetylglucosaminidase preparation (Ullrich *et al.*, 1979), and from the phosphomannose-specific receptors by which fibroblasts (Ullrich *et al.*, 1978a) and hepatocytes (Ullrich *et al.*, 1978b, 1979) recognize this enzyme. Thus asialo-orosomucoid and galactose, which block the uptake via the galactose/*N*-acetylgalactosamine receptor, and mannose 6-phosphate, a potent inhibitor for the uptake via the phosphomannose receptor, had no effect. In contrast with the phosphomannose receptor, which has a higher affinity for phosphorylated than for non-phosphorylated mannans (Kaplan *et al.*, 1977), the mannose/*N*-acetylglucosamine receptor has apparently a higher affinity for mannans poor in phosphate.

Previous experiments had indicated that purified human urine α -*N*-acetylglucosaminidase contains multiple forms that become recognized by receptors with different specificities (Ullrich *et al.*, 1978b, 1979). For enzyme replacement therapy in lysosomal storage disorders it is important to predict in which organs and cell systems the infused enzyme will

localize. Since liver appears to be the major site of lysosomal-enzyme clearance (see the introduction), we compared the uptake of α -*N*-acetylglucosaminidase, β -*N*-acetylglucosaminidase and α -mannosidase by parenchymal and non-parenchymal liver cells. Each of the three enzymes becomes internalized by parenchymal and non-parenchymal cells. A calculation based on several assumptions which may be met under conditions *in vivo* only approximately indicates that the preferential localization in either parenchymal or non-parenchymal cells clearly depends on the enzyme. Such results underline the need for the development of techniques that allow an organ- or cell-type-specific targeting of lysosomal enzymes for enzyme replacement therapy.

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