Research Article

Receptor-Mediated and Fluid-Phase Transcytosis of Horseradish Peroxidase across Rat Hepatocytes

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Horseradish peroxidase (HRP) is often used as a fluid-phase marker to characterize endocytic and transcytotic processes. Likewise, it has been applied to investigate the mechanisms of biliary secretion of fluid in rat liver hepatocytes. However, HRP contains mannose residues and thus binds to mannose receptors (MRs) on liver cells, including hepatocytes. To study the role of MR-mediated endocytosis of HRP transport in hepatocytes, we determined the influence of the oligosaccharid mannan on HRP biliary secretion in the isolated perfused rat liver. A 1-minute pulse of HRP was applied followed by marker-free perfusion. HRP appeared in bile with biphasic kinetics: a first peak at 7 minutes and a second peak at 15 minutes after labeling. Perfusion with 0.8 mg/mL HRP in the presence of a twofold excess of mannan reduced the first peak by 41% without effect on the second one. Together with recently published data on MR expression in rat hepatocytes this demonstrates two different mechanisms for HRP transcytosis: a rapid, receptor-mediated transport and a slower fluid-phase transport.

1. Introduction

The liver plays a major role in the secretion of macromolecules into bile either by receptor-mediated or unspecific fluid-phase transcytosis. Although the liver is composed of parenchymal (hepatocytes) and nonparenchymal cells (endothelial cells, Kupffer cells, Ito cells), only hepatocytes are involved in bile formation and biliary secretion of blood-derived molecules. As isolated hepatocytes in culture do not form a polarized epithelial layer where the apical and basolateral surfaces are accessible at opposite sides, transcytosis studies are confined to in vivo experiments or to the isolated perfused rat liver [1, 2]. Using the latter system, biliary secretion of various ligands taken up by receptor-mediated and/or fluid-phase endocytosis has been investigated so far. In contrast to other polarized epithelial cells (intestine, kidney, placenta) receptor-mediated endocytic processes are mainly confined to the basolateral (sinusoidal) surface of hepatocytes [1]. One of these sinusoidal receptors is a receptor for galactose-containing glycoproteins, so-called asialo-glycoproteins. This galactose receptor or asialoglycoprotein receptor (ASGPR) clears defective asialoglycoproteins (e.g., asialoorosomucoid; ASOR) from the circulation by receptor-mediated endocytosis. The receptors recycle and ASOR is directed to lysosomes for degradation. However, about 0.5–3% of internalized ASOR is missorted into the transcytotic route and consequently secreted intact into bile [3, 4]. Interestingly, the kinetics and amount of biliary secretion of different fluid-phase markers such as inulin or dextrans (MW 2–70 kDa) differ considerably, an effect that is generally attributed to their different molecular weight and/or charge [5, 6]. Even more surprising, horseradish peroxidase (HRP), following short pulse labeling, has a biphasic appearance in bile: the first, faster peak is assumed to occur by a paracellular route, while the second, slower peak takes place by transcytosis [7]. Transcytosis of the latter peak has been demonstrated by its complete absence in the presence of microtubule depolymerizing drugs [8]. With respect to the fast HRP appearance in bile the following data argue against a paracellular pathway: we have previously shown that low-temperature perfusion (16°C) results in a delayed appearance of the first peak in bile as compared to perfusion at 37°C [9]. Furthermore, 2 minutes after HRP loading HRP containing vesicles were seen in the vicinity of the canalicular...
membrane [7]. Moreover, HRP is not an ideal fluid-phase marker as it is a mannose-containing glycoprotein and thus binds to mannose receptors (MR) on hepatic Kupffer and endothelial cells with high affinity [10–12]. In addition, HRP binds with low affinity (Km ~ 4 μM) to isolated hepatocytes [11, 13] and in these cells HRP uptake up to 0.2 mg/mL could be competed by mannan, indicative for receptor-mediated endocytosis via an MR. To study the potential role of hepatic MR on HRP biliary secretion we determined the influence of the mannose oligosaccharide mannan (MW ~100 kDa) on HRP transport in the isolated perfused rat liver.

2. Materials and Methods

2.1. Animals. Male Louvain rats (250–300 g) were obtained from a local breeding farm and were kept under a controlled lighting schedule with a 12-hour dark period. Animals had free access to food and water but were fasted overnight before the experiment. Before surgical procedures, rats were anesthetized by an intraperitoneal injection of urethane solution (1 mL/100 g body-weight).

2.2. Materials. All chemicals were purchased from Sigma Chem. Co (Vienna, Austria). FITC-dextran (70 kDa) was extensively dialyzed against Tris-buffered saline (pH 7.34) and finally against phosphate-buffered saline (pH 7.4) before use. Asialoorosomucoid (ASOR) was obtained by enzymatic desialylation of α1-acid glycoprotein [14]. ASOR was iodinated in the presence of iodogen (Pierce) to a specific activity of approximately 1.4 × 10⁶ cpm/μg ASOR.

2.3. Biliary Secretion of Markers in the Isolated Perfused Rat Liver. Following surgical removal, isolated rat livers were equilibrated for 30 minutes at 37°C by single-pass perfusion with Krebs-Henseleith bicarbonate buffer (KHB; containing 10 mM glucose and 20 μM taurocholic acid and gassed with a humidified mixture of 93% O₂/7% CO₂, pH 7.4) at a perfusion rate of 3 mL/min and g liver [15, 16]. Biliary secretion of the respective marker was analyzed by single-pass perfusion at 37°C. HRP (0.08 mg/mL, 0.8 mg/mL or 8 mg/mL KHB) or FITC-dextran (5 mg/mL KHB) was perfused through the liver for 1 minute at 37°C followed by marker-free perfusion for 40 minutes. Where indicated, a twofold access of mannan over HRP was included in the perfusion buffer. Single bile drops were collected and analyzed for the respective marker concentration. To determine the kinetics of biliary secretion of ¹²⁵I-ASOR at 37°C, the liver was perfused for 2 minutes with ¹²⁵I-ASOR (1.3–2.3 × 10⁵ cpm/mL KHB) followed by marker-free perfusion for 40 minutes. Single bile drops were collected and TCA-precipitable (intact ASOR) and TCA-soluble (degraded ASOR) counts in each bile drop were determined.

2.4. Marker Determination. FITC-fluorescence of FITC-dextran per biledrop was measured in a spectrofluorometer (FP-777, Jasco, Japan) using 495 nm and 515 nm as excitation and emission wavelengths, respectively. After subtraction of background fluorescence, values were converted to concentration (pg FITC-dextran/biledrop) with the help of a calibration curve. The enzymatic activity of HRP per bile drop was determined based on standard curve according to Marsh et al. [17]. Following perfusion with ¹²⁵I-ASOR single bile drops were subjected to precipitation with 10% TCA (final concentration) and after centrifugation (1500 × g, 15 minutes) radioactivity in the supernatant (TCA soluble degradation products) and pellet (TCA precipitable, i.e., intact ¹²⁵I-ASOR) were determined by γ-counting (1282-Compugamma CS, LKB).

2.5. Calculations. The bile flow was calculated by determining the time when each bile drop was formed normalized to 1 g liver (bile flow in μL/min and g liver). The amount of marker/bile drop was determined using the respective calibration curve (HRP, FITC-dextran) or radioactivity. As one bile drop corresponds to 8 μL bile, these data were converted to marker secretion (amount of marker/min and g liver) by division by 8 and multiplication with the respective bile flow. The appearance of the marker in bile was corrected for the dead space of the bile duct cannula. The cumulative HRP secretion was obtained by summing up the amount of HRP/bile drop over time. To analyze differences between the first and the second peak of HRP secretion, we defined the cumulative amount that was secreted between 0 and 10 minutes to correspond to the 1st HRP peak (secretion maximum 6–8 minutes). This amount was then subtracted from the total amount of HRP secreted within 40 minutes to obtain the total amount secreted during the 2nd HRP peak (secretion maximum 15 minutes).

3. Results

3.1. Kinetics of Biliary Secretion of the Fluid-Phase Marker FITC-Dextran and the Ligand, Asialoorosomucoid (ASOR). For comparison, the secretion kinetics of a bona fide fluid-phase marker, FITC-dextran, and a galactose-terminated ligand, asialoorosomucoid (ASOR), were analyzed. FITC-dextran (MW 70 kDa; 5 mg/mL) was added to the perfusate and perfused through the liver for 1 minute (= pulse) under single-pass conditions. To maintain constant bile flow 20 μM taurocholic acid was always included in the perfusion buffer. Perfusion was then continued in the absence of marker for up to 40 minutes. The concentration of the marker was analyzed in single bile drops and data were related to bile flow and perfused through the liver for 1 minute (= pulse) under single-pass conditions. The bile flow was calculated by determining the time when each bile drop was formed normalized to 1 g liver (bile flow in μL/min and g liver). The amount of marker/bile drop was determined using the respective calibration curve (HRP, FITC-dextran) or radioactivity. As one bile drop corresponds to 8 μL bile, these data were converted to marker secretion (amount of marker/min and g liver) by division by 8 and multiplication with the respective bile flow. The appearance of the marker in bile was corrected for the dead space of the bile duct cannula. The cumulative HRP secretion was obtained by summing up the amount of HRP/bile drop over time. To analyze differences between the first and the second peak of HRP secretion, we defined the cumulative amount that was secreted between 0 and 10 minutes to correspond to the 1st HRP peak (secretion maximum 6–8 minutes). This amount was then subtracted from the total amount of HRP secreted within 40 minutes to obtain the total amount secreted during the 2nd HRP peak (secretion maximum 15 minutes).
the perfusate and after ligand-receptor dissociation in early endosomes the receptor is recycled and ASOR is sorted to lysosomes [18, 19]. The appearance of ASOR degradation products in bile reflects the kinetics of arrival in lysosomes. About 90% of ASOR degradation products are released into the perfusate and the remainder into bile [3]. Since 0.5%–3% of internalized ASOR is missorted and secreted intact into bile, determination of TCA-precipitable and TCA-soluble radioactivity in bile correlates with transcytosis and transport to lysosomes, respectively [3, 4]. Biliary secretion of ASOR at 37°C was analyzed following a 2-minute pulse of 123I-ASOR with subsequent marker-free perfusion of the liver. Single bile drops were collected and TCA-precipitable (intact) and TCA-soluble (degraded) radioactivity therein were determined (Figure 1(c)). Intact 123I-ASOR appeared in bile within 6 minutes after the pulse, while maximum biliary secretion was found about 17 minutes after uptake. 15 minutes after internalization TCA-soluble counts appeared in bile, corresponding to ASOR arrival in lysosomes, immediate degradation and release of degradation products into the perfusate (not shown; [3, 4]) and bile. The bile flow (Figure 1(d)) remained constant at 1.8 μL/g liver and min throughout this perfusion. Taken together, biliary secretion of the fluid-phase marker FITC-dextran is faster than the ligand ASOR.

3.2. Kinetics of Biliary Secretion of HRP and the Effect of Mannan on HRP Secretion. HRP (0.8 mg/mL KHB) was added to the perfusion medium for 1 minute followed by marker-free perfusion (Figure 2(a)). Although HRP appeared in bile roughly at the same time as FITC-dextran, the secretion rate revealed two peaks at 7 minutes and 15 minutes, respectively. These two peaks were seen irrespective of the HRP concentration perfused through the liver (0.08; 0.8; 8 mg/mL) and are in agreement with data from different laboratories [7, 8, 20, 21]. The bile flow, shown in Figure 2(b), remained constant at 1.6 μL/g liver and min throughout this perfusion. The cumulative secretion is shown in Figure 2(c). Comparison of HRP secretion with secretion of FITC-dextran and ASOR revealed that HRP exhibits a similar kinetics as the fluid-phase marker (1st HRP peak) as well as that of the ligand (2nd HRP peak).

Next, we analyzed the influence of 1.6 mg/mL mannan on HRP secretion (Figure 3). Similar to the absence of mannan (compare to Figure 2) two peaks of secreted HRP were seen, at about 8 minutes and 16 minutes, respectively (Figure 3(a)). The bile flow was maintained at about 1.5 μL/min and g liver (Figure 3(b)). Figure 3(c) demonstrates that the total cumulative secretion was reduced in the presence of mannan as compared to its absence (compare to Figure 2(c)). To differentiate whether mannan
influenced rather the first or the second peak, the amount of HRP being secreted between 0 and 10 minutes (i.e., during the fast, first peak) and between 10 minutes and 40 minutes (i.e., during the slow, second peak) was analyzed. As depicted in Figure 4, the first HRP peak was reduced by 41% by mannan without any influence on the second peak. When HRP at 0.08 mg/mL was applied in the perfusate, the first peak was also decreased by mannan by 32% while the second peak was slightly increased by 14% (data not shown). However, due to the low HRP concentration in single bile drops individual experiments varied considerable. When the HRP concentration in the perfusate was raised to 8 mg/mL, no significant effect of 16 mg/mL mannan could be seen (data not shown). These data demonstrate that the fast pathway of HRP entry into bile has to occur at least in part by a receptor-mediated transcellular route. Nevertheless, the kinetics of this route clearly differs from that of the ligand ASOR (see Figure 1(c)).

4. Discussion

4.1. Characteristics of Liver MR. In our study, we have observed an effect of mannan on the first peak of HRP-secretion that indicates an interaction of HRP with MR on hepatocytes. The MR (180 kDa) was first isolated from
Data shown are the mean calculated for 0–10 minutes (1st peak) and 10–40 minutes (2nd peak). Asterisk indicates significant differences at \( P \leq .05 \).

Figure 4: Effect of mannan on cumulative HRP secretion. The cumulative secretion from the experiments shown in Figure 2 (absence of mannan) and Figure 3 (presence of mannan) was calculated for 0–10 minutes (1st peak) and 10–40 minutes (2nd peak). Data shown are the mean ± SEM from five and four perfusions, respectively. Note that only the first peak (0–10 minutes) was reduced in the presence of mannan. Asterisk indicates significant differences at \( P \leq .05 \).

rat liver Kupffer cells [22] and binds glycoproteins with, for example, terminal mannose or fucose groups in a Ca-dependent manner. The MR is found on monocyte-derived and tissue macrophages, cultured dendritic cells, on hepatic sinusoidal and lymphatic endothelial cells in the small intestine [23]. Its main function is the clearance of many serum glycoproteins, lysosomal enzymes, and denatured collagen containing the respective sugar moieties resulting in their rapid degradation [24–28]. Rat liver endothelial cells exhibit a high MR surface density and high affinity (Km about 30–60 nM) for ligands such as ovalbumin [28] or N-acetylgalactosamine containing glycoproteins [29]. MRs are taken up by clathrin-mediated endocytosis due to a conserved lysine residue and a dihydrophobic motif in their short cytoplasmic tail [30, 31]. Internalized MR-ligand complexes dissociate in the low pH environment of endosomes [32] resulting in receptor recycling and ligand transport to and degradation in lysosomes [28, 30].

Apart from being localized to Kupffer and endothelial cells receptors for mannosylated glycoproteins were also described in liver parenchymal cells [11, 33]. Quantitative data from electron microscopy autoriadiographic studies revealed that a minor fraction of mannosylated RNase was found in hepatocytes [34]. Similarly, aglucerase was taken up by hepatocytes in a mannose-dependent fashion [35]. Using mannosylated gold particles, Kempka and Kolb-Bachofen demonstrated binding of the marker in coated pits followed by uptake, transcytosis, and secretion into bile by rat hepatocytes in situ [12]. Although these events in hepatocytes were rare as compared to liver sinusoidal cells, they were not completely absent. Using isolated liver parenchymal cells, binding, uptake, and intracellular routing to lysosomes of invertase was shown that could be blocked by mannose [36]. Collectively, these data support the presence of an MR on the sinusoidal surface of rat hepatocytes. However, the MR on hepatocytes is clearly distinct from the MR on Kupffer and endothelial cells and has not been identified, so far. Antibodies specific for the 180 kDa MR on endothelial cells did not reveal a protein with similar molecular weight in hepatocytes [37]. Binding of HRP to isolated hepatocytes at 4°C was of low affinity thus preventing the identification of a specific plasma membrane protein [13]. However, HRP uptake at 37°C was saturable with a Km of 8.3 mg/L and could be inhibited by mannan with an apparent KI of 2.5 mg/L [13] clearly demonstrating the involvement of MR in HRP endocytosis in hepatocytes.

4.2. Endocytic Compartments Involved in Receptor-Mediated and Fluid-Phase Transcytosis. Receptor-mediated endocytosis can take place via clathrin-coated pits and vesicles (e.g., ASOR, polymeric IgA), via caveolae (e.g., albumin in endothelial cells), and other less characterized mechanisms [38, 39]. The prevalence of the respective uptake mechanism depends on the ligand and cell type. Irrespective of the uptake mechanism by which each endocytic vesicle is formed, it contains extracellular fluid and extracellular material (proteins, ions, etc.). Consequently, fluid-phase endocytosis occurs concomitantly with receptor-mediated endocytosis. However, the contribution of distinct endocytosis mechanisms to fluid-phase uptake depends on the cell type under investigation. The kinetics of transfer of different markers, be they ligands or fluid-phase markers, do not necessarily allow a prediction of their intracellular route. FITC-dextran (fluid phase marker) and ASOR (ligand) utilize the same endocytic subcompartments on their transcellular route from the sinusoidal to the canalicular plasma membrane [40]. This has been shown by low-temperature (16°C) perfusion of isolated perfused rat livers and by subcellular liver fractionation [40]. Under this condition FITC-dextran and ASOR accumulate in early endosomes resulting in inhibition of transcytosis of both molecules and inhibition of lysosomal degradation of ASOR. Nevertheless, they are secreted into bile with completely different kinetics (Figures 1(a) and 1(c)).

Similar to the transcytosis of ASOR and FITC-dextran, low-temperature perfusion blocks the slow pathway of HRP secretion indicative for involvement of the same endocytic compartments (early endosomes) during transport to the canalicular membrane. Consequently, a part of HRP has to be taken up into typical early endosomes (that accumulate marker at 16°C), is then directed into transcytotic vesicles, and appears in bile at about 15 minutes (Figure 5). In contrast, a proportion of HRP is internalized via an MR (mannan competeable uptake) into endocytic compartments distinct from ASOR-containing endosomes. Such a distinct fast transcellular pathway is supported by the observation that HRP appearance in bile during 16°C perfusion is only delayed but not blocked [9] and that HRP-containing endosomes are observed within 2 minutes in the vicinity of the canalicular membrane [7]. Evidence for distinct internalization pathways has also been obtained for other biliary secreted markers. Albumin and ASOR have been localized in distinct endocytic compartments early after uptake in isolated hepatocytes at low temperature [41].

It is still a matter of debate whether the fast HRP appearance in bile is (additionally) due to paracellular transport as supported by stimulation of this peak under
postcholestatic conditions and by reagents that lead to intra-hepatic cholestasis [8, 9, 42]. All these treatments increase the permeability of the tight junctions and could therefore explain the stimulatory effect on the first HRP peak [42, 43]. However, the data presented in this investigation clearly demonstrate that a least part of the first HRP peak is reduced by mannann and is thus accounted for by receptor-mediated transcytosis. It may be speculated that mannann reduces the paracellular permeability. However, since mannose receptors are only involved in the binding and subsequent endocytosis of mannann containing glycoproteins to/into cells, it can be assumed that mannann has no influence on the paracellular permeability, though this has not been proven. We cannot think of an experimental setup to unequivocally differentiate between the proportion of the transcellular and paracellular part of the 1st peak. Such a setup is required to determine the effect of mannann treatment on the paracellular permeability.

4.3. Actual HRP Concentration in the Perfusate. Kupffer cells and endothelial cells have an up to 10-times higher endocytic activity of fluid-phase marker uptake per se [34, 35, 44] and, in addition, mannose-containing glycoproteins are internalized by high affinity MR into endothelial cells at a very fast endocytic rate [28, 45]. Consequently, the actual HRP concentration that will be in contact with hepatocytes in the isolated perfused rat liver is unknown. It can be assumed to be much lower as the amount of HRP added to the perfusion buffer due HRP removal by endothelial and Kupffer cells. This may explain the discrepancies between our results and data by Yamaguchi et al. [13]. These authors observed saturation of HRP uptake in isolated hepatocytes already at 0.2 mg/mL HRP. In contrast, we could demonstrate mannann inhibitable HRP secretion at 0.8 mg/mL. Thus, when HRP is applied at concentrations ranging between 1 mg/mL and 10 mg/mL as in morphological and perfusion studies [7, 8, 20, 21, 46] the proportion due to receptor-mediated endocytosis cannot be ignored, although at such HRP concentrations uptake by isolated hepatocytes is mainly by a fluid-phase mechanism.

4.4. Role of Hepatocytes and the Biliary Epithelium in Bile Formation and Composition. Bile is produced by hepatocytes at their canalicular surface due to activity of many solute transporters [47]. The main bile constituent is water (about 96%). The remainder is composed of bile acids (67%), phospholipids (22%), cholesterol (5%), and proteins (5%). The canalicular bile then passes through the bile ducts and is collected in the gallbladder for regulated secretion into the intestinal tract [48]. Ductular epithelial cells modify the canalicular bile by a series of secretory and reabsorptive processes resulting in its alkalinization and concentration. Thus, ductular/gallbladder bile has a lower water content (about 87%) and a higher concentration of all the constituents listed above as canalicular bile. Many biliary proteins (polymeric IgA) are secreted via receptor-mediated transcytosis while others (albumin, IgG) reach bile by a fluid-phase mechanism. Though it has been shown that the total protein concentration is higher in ductular/gallbladder than in canalicular bile due to water absorption, additional absorptive/secretory processes may affect the concentration of individual proteins [49]. With respect to HRP we may speculate that this marker is endocytosed by ductular cells by a fluid-phase mechanism as shown in isolated rat bile duct epithelial cells [50, 51]. Such processes may result in a reduction of the amount of HRP in ductular bile. Thus, the actual amount of HRP secreted by hepatocytes may be higher. How ductural endocytosis may affect the concentration of the first and second HRP peak is unknown and cannot be taken into consideration. In any case, we here demonstrate that HRP secretion in the isolated perfused rat liver can be inhibited by mannann indicative for the involvement of MR in HRP uptake by hepatocytes.

5. Conclusions

The potential pathways for HRP secretion into bile are summarized in Figure 5: (1) a receptor-mediated, fast, transcellular route, (2) a slow, fluid-phase transcytotic route and (3) most likely also a fast paracellular route that is increased by intra- and extrahepatic cholestasis. Based on previous data [8, 52] the slow pathway is identical to that taken by polymeric IgA and ASOR, that is, clathrin-mediated uptake into early endosomes where sorting into transcytotic vesicles takes place followed by biliary secretion. This pathway is blocked by 16°C in early endosomes and is entirely dependent on microtubules [8, 9, 52]. In contrast, the fast transcellular route is microtubule-independent and insensitive to inhibition by low temperature [8, 9]. This pathway may involve clathrin-coated pits and vesicles similar to the uptake of mannose containing glycoproteins in dendritic cells [53].

Figure 5: Scheme of potential pathways involved in HRP secretion into bile. Compartments labeled by HRP are indicated by blue color. Although HRP is also directed to lysosomes, only those endosomes involved in transcytosis are shown in blue. MRs (yellow bars) presumably recycle back to the sinusoidal plasma membrane.
Abbreviations

HRP: Horseradish peroxidase
ASGPR: Asialoglycoprotein receptor
ASOR: Asialoorosomucoid
KHB: Krebs-Henseleith bicarbonate buffer
MR: Mannose receptors.

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