Liver Endothelium Mediates the Hepatocyte’s Uptake of Ceruloplasmin

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Abstract. The mode of transport of ceruloplasmin (CP) into the liver was investigated in fractionated liver cell suspensions. Incubation of 125I-CP at 4°C with these different fractions led to its binding only to endothelial cells but not Kupffer cells and hepatocytes. Incubation at 37°C led to rapid uptake of 125I-CP by endothelium, but cell-associated radioactivity declined after 15 min, which suggests the release of the labeled substance. Internalization was confirmed by incubation of endothelium with double-labeled CP (1H label on sialic acid and 125I on the protein part). We conclude that in the liver, CP is first recognized and taken up by endothelial cells that are endowed with appropriate surface receptors for the protein. Endothelium then modifies the molecule by desialation to expose the penultimate galactosyl residues. The modified molecule is then released, recognized, and taken up by hepatocytes through their membrane galactosyl-recognition system. These findings are consistent with the role of endothelium as an active mediator of molecular transport between blood and tissue, and further assign a biological role for the galactosyl-recognition system in hepatocytes.

Materials and Methods

Materials

CP (Sigma Chemical Co., St. Louis, MO) was purified in our laboratory on a Sephadex G-200 column and its purity was then demonstrated by polyacrylamide gel electrophoresis (PAGE). Lyophilized metrizamide (grade 1), collagenase type IV, bovine serum albumin (fraction V powder, BSA), and neuraminidase from Clostridium perfringens attached to beaded agarose were also obtained from Sigma Chemical Co. Carrier-free Na125I, Na3H1, and Bolton-Hunter reagent were obtained from ICN Pharmaceuticals, Inc. (Irvine, CA) and immobilized lactoperoxidase from Worthington Biochemical Corp. (Freehold, NJ).

Fractionation of Liver Cell Suspensions

Liver cell suspensions were prepared from male Sprague-Dawley rats (200–250 g) by a collagenase perfusion method that has been described in detail (8, 18, 19). Fractionation of these suspensions was done first on metrizamide gradients by a modified method of Seglen (18), also described previously in detail (19). This fractionation yielded two cellular fractions, one that consisted of 99% hepatocytes (identified in SEM and TEM by counting 1,000 cells) and only 1% nonparenchymal cells (Kupffer and endothelial cells). This suggested that the released molecule was modified in the endothelium by desialation. Desialation was confirmed by incubation of endothelium with double-labeled CP (1H label on sialic acid and 125I on the protein part). We conclude that in the liver, CP is first recognized and taken up by endothelial cells that are endowed with appropriate surface receptors for the protein. Endothelium then modifies the molecule by desialation to expose the penultimate galactosyl residues. The modified molecule is then released, recognized, and taken up by hepatocytes through their membrane galactosyl-recognition system. These findings are consistent with the role of endothelium as an active mediator of molecular transport between blood and tissue, and further assign a biological role for the galactosyl-recognition system in hepatocytes.

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Beckman J6-B standard elutriation rotor (4.2 ml) using a J2-21 centrifuge (30). Before cell loading, the rotor was eluted with PBS at a flow rate of 11 ml/min. The rotor was then loaded with the cell suspension in PBS at a concentration of 10⁷/ml using a total volume of no more than 5 ml, maintaining the same flow rate. After the cell loading, elutriation was carried out at 750 g (2,500 rpm) at 20°C. The flow rate was increased to 20 ml/min, and 250 ml of eluant fluid was collected. This fraction contained 95-99% endothelium (Table 1) as determined by examination of 1,000 cells (for each differential cell count) in SEM and TEM preparations (see the text). This was further confirmed by the reaction with factor VIII antibody (using an indirect method [23]). The viability was ~99%. The second fraction consisted of >90% Kupffer cells as identified by SEM and TEM. The contaminant was mostly endothelial cells but also a few bile epithelial cells. Viability of recovered cells was above 97%.

Radioisotope Labeling Methods

Iodination of CP was done by the lactoperoxidase method as described (4, 8). The final specific activity of the labeled protein was 0.25 mCi/mg. CP was also double-labeled with ³H (sialic acid residues) and ¹²⁵I (protein moiety) as described elsewhere (11). ³H labeling was done by the consecutive oxidation-reduction method of Van Lenten and Ashwell (27). CP (1 mg/ml) was dissolved in 0.1 M sodium acetate that contained 0.15 M NaCl (pH 5.6). This was oxidized by incubation with 0.24 ml of 0.012 M NaIO₄ for 10 min at 0°C. Oxidation was stopped by the addition of excess ethylene glycol (1 ml), and the solution was dialyzed overnight at 4°C against PBS, pH 7.4. Periodate consumption, determined by the method of Dyer (5), indicated more than 1.8 mol of periodate consumed per mole of sialic acid, remarkably close to 2 mol per mole which is theoretically ideal. Tritium was then introduced into sialic acid residues by adding 0.1 mg tritiated NaBH₄ dissolved in 0.02 ml of 0.01 M NaOH. After mixing, the solution was warmed to room temperature, and the reduction was allowed to continue for 30 min with stirring. To ensure the completeness of reduction, 0.3 mg of nonradioactive KBH₄ was added and the incubation was continued for another 30 min. Excess borohydride was removed by dialysis against 0.1 M Na acetate and 0.15 M NaCl (pH 5.6) for several hours before exhaustive dialysis against PBS (pH 7.4). Subsequently hydrolysis of the sialic acid (27) by either sulfic acid or enzymatic method (neuraminidase) demonstrated that, respectively, 99.5% or 98.7% of the label was sialic acid.

This tritiated CP was then iodinated by using the Bolton-Hunter reagent (3) since the usual peroxidation method could remove the ³H label from sialic acid. To obtain maximum labeling, tritiated CP was dialyzed overnight against 0.1 M borate buffer (pH 8.5) and to this solution 1 mCi of Bolton-Hunter reagent in 0.1 M borate buffer was added and incubated for 15 min at 0°C. Unlabeled reagent was reacted with 0.5 ml of 0.2 M glycin in 0.1 M borate buffer for 15 min at 0°C. The preparation was passed through a G-25 Sephadex column (0.7 x 10 cm) to remove glycine and unattached Bolton-Hunter reagent. The final specific activities of ³H and ¹²⁵I were 0.12 mCi and 0.11 mCi per milligram of protein.

Removal of Sialic Acid

CP was desialylated by incubating 10 mg/ml of the protein with 0.5 µg of neuraminidase attached to beaded agarose (29) in a buffer (pH 5.5) that contained 0.1 M sodium acetate with 5 mM calcium chloride for 24 h at room temperature in a mechanical shaker. Neuraminidase was then removed by centrifugation. Asialoceruloplasmin (ASCP) was separated from free sialic acid by passing through the column of Sephadex G-25 (2 x 25 cm). The removal of sialic acid was confirmed by the thiobarbituric acid method (26) and calculation indicated that more than 90% of the sialic acid content of the molecule was removed. Incubations

Incubations were done in microcentrifuge tubes. Suspensions of endothelium or hepatocyte fractions (1 x 10⁶ cells/tube) in Dulbecco's PBS, pH 7.0, supplemented with 0.5% BSA were incubated with the radiolabeled ligand in a total volume of 250 µl at the desired temperature for the desired period. The reaction was terminated by the addition of 1 ml of cold PBS. Nonspecific binding was determined in the presence of a 100-fold excess unlabeled ligand. The nonspecific binding was subtracted from total binding, and the result was expressed as specific binding. The tubes were centrifuged in a microcentrifuge for 10 s. The pellets were washed twice with 1 ml of cold PBS. The washed cell pellets and the combined supernates were counted for the radioactivities.

Separation of Surface-bound and Internalized Ligand

This was done by acetic acid dissociation of surface-bound ligand according to the method of Haigler et al. (7). Briefly, after incubation with the ligand, cells were washed three times in PBS and incubated for 6 min at 4°C with 0.7 ml of equal proportions of 0.2 M acetic acid and 0.5 M NaCl (pH 2.5). The cells were then centrifuged in a microcentrifuge, and the supernate was collected. The cells were further incubated for another 6 min with 0.4 ml of the same acid solution, further centrifuged, and the supernate was again collected. The combined supernates were counted to determine the surface-bound ligand, while the cell pellet was counted after lysis with 1 N NaOH to determine the internalized ligand.

Results

The Binding and Uptake of ¹²⁵I-CP by Liver Cell Fractions

Incubation of liver cell fractions with ¹²⁵I-CP at 4°C led to the binding of CP only by the endothelium. Hepatocytes and Kupffer cells did not bind ¹²⁵I-CP significantly at 4°C (Fig. 1). The binding of the endothelium fraction was ligand concentration-dependent, and from the Scatchard analysis (17), the maximum binding was calculated to be ~347 fmol per 10⁶ cell particles.

Figure 1. The binding of ¹²⁵I-CP to endothelium, Kupffer cell, and hepatocyte fractions of the liver cell suspension. Cells in each fraction (1 x 10⁶) were incubated at 4°C for 60 min with an increasing concentration of ¹²⁵I-CP in 250 µl (total volume) of Dulbecco's PBS that contained 0.5% BSA. Inhibition was done in the presence of 100-fold excess (4 mg/ml) of unlabeled CP. Specific binding was calculated by subtracting nonspecific binding from total binding. Inset shows a Scatchard plot constructed from the specific binding data for the endothelium. Minimal binding was noted by hepatocytes and Kupffer cells and can be explained by contamination with endothelial cells.
cells with dissociation constant ($K_d$) of $1 \times 10^{-7}$ M. The number of receptors per cell was $2.1 \times 10^5$. The specificity of the binding of $^{125}$I-CP was demonstrated by inhibition in the presence of a 100-fold excess (4 mg/ml) native CP. The magnitude of specific binding of $^{125}$I-CP was a function of cell number (Fig. 2).

Specific binding of $^{125}$I-CP to the endothelium as a function of time is shown in Fig. 3. At 4°C the binding was saturable, reaching a steady-state level between 5 and 30 min (data not shown). At 37°C (Fig. 3), however, the maximum uptake by endothelium occurred after 15 min. This was considerably higher than the binding plateau at 4°C (135 ng vs 35 ng per 10^6 cells). Cell-associated radioactivity somewhat declined thereafter, which suggests the release of the label from the cells at a rate exceeding that of its uptake. To assure that this decline was not the result of degradation of CP through proteolysis, the proteins in the supernate were precipitated with 20% trichloroacetic acid (TCA), and the precipitant as well as the supernate were counted for their radioactivity. At no time did the TCA nonprecipitable (soluble) radioactivity exceed 3% of total radioactivity, which suggests the absence of significant CP degradation. Moreover, the supernate was subjected to column chromatography on either Sephadex G-25 or G-200. The radioactivity remained in a single peak corresponding to that of CP. Cell viability at the end of the 90-min incubation was unchanged. Dissociation of total ligand uptake into surface-bound and internalized compartments is also shown in Fig. 3. The internalized ligand as a function of time followed a curve parallel to total uptake, reaching a maximum after 15 min and somewhat declining thereafter. Surface-bound ligand, on the other hand, reached a maximum within 5 min and remained stable or even declined slightly.

Further evidence for binding and release of $^{125}$I-CP by endothelium was demonstrated in an experiment in which endothelium was first incubated with labeled ligand at 4°C for 30 min. Cells were then washed and transferred to 37°C for various periods. After completion of incubation, cells were centrifuged and radioactivity was counted in both cells and the supernate. Cell-bound radioactivity, which after incubation at 4°C had reached the same magnitude as in Fig. 1, began to decline as a function of time after the transfer of cells to 37°C (Fig. 4). A reciprocal increase in the radioactivity of the supernate indicated the release of radioactivity.

To assure the absence of proteolysis, the proteins in the supernate were precipitated with 20% TCA and the precipitant as well as the supernate were counted for their radioac-

Figure 2. Effect of cell number on the specific binding of $^{125}$I-CP to the endothelial-rich fraction. Variable numbers of endothelial cells (0.5-2.0 $\times 10^6$ cells per 250 $\mu$l) were incubated with 10 $\mu$g of $^{125}$I-CP for 60 min at 4°C. Specific binding was calculated as before. The binding is a linear function of the cell number.

Figure 3. Uptake of $^{125}$I-CP to endothelium fraction at 37°C. Cells (1 $\times 10^6$) were incubated with 10 $\mu$g of $^{125}$I-CP at 37°C for indicated periods in a final volume of 250 $\mu$l. The reaction was terminated by the addition of 1 ml of cold PBS after the desired period. Inhibition was done in the presence of unlabeled CP (4 mg/ml). Specific uptake is shown as a function of time. Total uptake reaches a peak within 15 min and then appears to decline somewhat, which suggests the release of the label at a rate higher than that of uptake. Separation of radioactivity into surface-bound and internalized fractions was done as described in the text. The internalized fraction reaches a peak within 15 min and then begins to decline in a curve that parallels that of total uptake. The surface bound fraction reaches a peak within 5 min and then slightly declines, which suggests that some down regulation might have occurred.

Figure 4. Endothelial cells (1 $\times 10^6$) were incubated with 10 $\mu$g $^{125}$I-CP at 4°C for 30 min. The cells were then washed three times in PBS and further incubated at 37°C for various periods. The cell-associated radioactivity at time zero (surface-bound) declines with time, and this decline is associated with a corresponding increase in the radioactivity in the supernate.
ativity. At no time did the TCA nonprecipitable (soluble) radioactivity exceed 3%, which suggests the absence of significant CP degradation. Moreover, the supernate was subjected to column chromatography on either Sephadex G-25 or G-200. The radioactivity remained in a single peak corresponding to that of CP. No significant specific uptake of CP by hepatocytes or Kupffer cells was noted at 37°C either (data not shown).

The Binding Capacity of the Supernate

Incubation of the supernate, "conditioned" by previous incubation with endothelium, with fresh target hepatocytes now led to the binding of the radioactive ligand in the supernate to cells (Fig. 5), which suggests that the radioactive ligand released from the endothelium might have acquired a new site recognizable by hepatocytes. This binding was not inhibited by excess unlabeled CP but was significantly inhibited by the galactosyl-containing molecules ASCP (Fig. 5), galactosyl-BSA synthesized in our laboratory (9), as well as asialofetuin (25).

Desialation of CP by Endothelium

Double-labeled CP (tritiated sialic acid, iodinated protein moiety) was incubated with endothelium fraction at 37°C for various periods, and the cell-associated radioactivities were counted separately. The results, shown in Fig. 6, demonstrated that for the first 15 min the curves for both ³H and ¹²⁵I were overlapping, which indicated the uptake of the molecule that contained both labels. Thereafter, the two radioactivities dissociated with the ¹²⁵I curve slightly declined in a pattern similar to Fig. 3, while the ³H curve remained stable or even slightly increased. The data provided evidence for desialization of CP molecule by the endothelium.

Mixed Cell Experiments

The results described above suggested that CP is removed by endothelium and then released only to be taken up by hepatocytes (i.e., the endothelium mediates the uptake of CP by hepatocytes). To further evaluate this, mixtures of endothelium and hepatocytes in equal ratios (1 x 10⁶ cells each) were incubated at 37°C with the radiolabeled CP for various periods of time. The mixture was then refractionated, and the cell-associated radioactivity was counted separately for each fraction. The results are shown in Figs. 7 and 8. It will be noted
and the small amount of binding observed could be explained by contamination with a small number of endothelial cells. During the same period, endothelial cell number. That only a fraction of CP present in the medium is taken up by the cells may be due to down regulation of receptor as a consequence of continued exposure to the ligand (25). It is also possible that the receptor and ligand is transported in a complex form by the endothelium precluding receptor recycling. This area deserves further study.

By fractionating the ligand taken up by cells into surface-bound and internalized fractions, we have further confirmed that the protein is indeed internalized by endothelial cells. This also confirms our previous observations using the minibead probe. In addition, we have demonstrated that the internalized ligand is then released by the endothelium into the medium, probably through a process of transcytosis. We have obtained confirmatory evidence for transcytosis of CP (10) by in situ perfusion of liver with colloidal gold–labeled CP. The label binds to the luminal surface of liver sinus endothelium at 4°C and is internalized at 37°C. Morphometric studies indicate that the probes then move via a vesicular transport system and are externalized on the abluminal side. During this process, the endothelium appears to desialate the molecule. This is suggested by dissociation of $^3$H and $^{125}$I after incubation with the endothelium in those experiments in which sialic acid residues were labeled with $^3$H while the protein part of the molecule was iodinated. Additional support for desialation of the molecule was obtained by electrophoresis of the supernate, which demonstrates the generation of a radioactive band co-migrating with ASCP. This evidence has been presented elsewhere (25). Desialation can expose the penultimate galactosyl residues of the glycoprotein. Since hepatocyte membrane is known to possess specific receptors for galactosyl termini of glycoproteins, the release of desialated glycoprotein can lead to its binding and uptake by hepatocytes. Thus, the endothelium-conditioned molecule acquires the capacity to bind to fresh target hepatocytes as we have demonstrated here.

That the galactosyl receptors of hepatocyte membrane may be involved in the binding of endothelium—conditioned molecules is suggested by the lack of inhibition of hepatocyte binding with excess native CP, but the presence of inhibition with galactosyl-terminating molecules (ASCP, asialofetuin, galactosyl-BSA).

Incubation of radioactive ligand with a mixture of endothelial cells and hepatocytes indicated that the endothelial cell processing of the ligand may be necessary for its uptake by hepatocytes: cell-associated radioactivity rapidly increased in the endothelial cell fraction and this was followed by a slow drop and a reciprocal slow increase in the hepatocyte fraction. Moreover, in this system, the uptake of ligand by both fractions was inhibited in the presence of excess unlabeled CP, whereas the uptake of endothelium-processed CP by hepatocyte was not.

Our work, therefore, suggests a physiological function for galactosyl receptors of hepatocyte membrane. These studies further emphasize the function of endothelial barrier in the uptake of proteins. Similar endothelial transcytosis has been reported for another plasma protein, low density lipoprotein, in aorta (28), for lactoferrin in the liver (13), and by our own group for transferrin in the bone marrow (20). We have also observed endothelial mediation of transport in the liver for three other plasma proteins thus far studied: insulin, transferrin, and transcobalamin II (19, 21, 22). This mode of tissue uptake of plasma proteins may, therefore, be a general phenomenon and, if so, galactosyl receptors of hepatocytes could be considered as universal acceptor for many glycoproteins.

Further support for this view is provided by the work of...
Regoezzi et al. (15, 16) who have used infusion of human asialotransferrin type 3 in vivo as well as binding studies in vitro with purified rat liver plasma membrane. They demonstrated that while the liver uptake of asialotransferrin is via galactosyl receptors, competition with native protein can reduce the half-life of the ligand, which suggests that both receptors for native glycoprotein and asialoglycoprotein are needed for the uptake. They then concluded that diacytosis of asialotransferrin could explain their data.

Modification of the ligand, as suggested by our work, is further in line with the work of Greenspan and St. Clair (6) who have described the modification of low density lipoprotein during its passage through the skin fibroblasts. This process, called retroendocytosis, is very similar to modification of CP during its transcytosis across liver endothelium as described here. Thus, cellular modification of proteins for subsequent use may have other examples in cell biology.

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