Identification of a heparin-releasable hepatic lipase binding protein from rat liver

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Hepatic lipase (HL) plays a key role in the metabolism of several lipoproteins. Metabolically active HL is bound in liver parenchymal cells to specific binding sites. We studied the nature of the HL binding in rat liver. Rat livers were perfused with heparin, which lead to a loss of 80 % of the HL binding capacity of the liver. The heparin-containing perfusates possessed HL binding capacity, determined by slot-blot assay. The perfusates were loaded on to a heparin-Sepharose column and eluted with a linear salt gradient (0.2–1 M). HL binding activity, assessed by a slot-blot binding assay, eluted both at 0.3 M and at 0.8 M NaCl. A 0.5 M NaCl eluate was used to further characterize the HL binding activity. In this fraction the major protein had a molecular mass of 70 kDa. The fraction showed saturable HL binding in a solid-phase binding assay. Cross-linking of the 0.5 M NaCl fraction to ^{125}I-labelled HL yielded a complex of 130 kDa, suggesting the cross-linking of the 57 kDa ^{125}I-labelled HL to a protein of about 73 kDa. We concluded that heparin releases a protein of about 73 kDa from rat liver, which associates with HL. This protein may represent the HL binding site in liver.

INTRODUCTION

Hepatic lipase (HL) is found in liver as an extracellularly located enzyme. The enzyme is synthesized in liver parenchymal cells [1] and is subsequently bound to the parenchymal cell microvilli [2]. Because of its extracellular localization, HL is able to play an important role in the metabolism of lipoproteins. A function in the metabolism of high-density lipoproteins (HDL), intermediate-density lipoproteins (IDL) and chylomicron remnants [3–9] has been suggested. It has been proposed that, besides its catalytical activity, HL acts as a ligand for lipoprotein binding to receptors [10].

Recently, we showed that the binding capacity of the liver for HL is limited [11], suggesting the presence of (a) specific HL binding site(s). The specificity of the binding sites was further substantiated by the finding that, although HL binding capacity is fully utilized, the structurally related lipoprotein lipase (LPL) can still be bound to the liver. In addition, LPL was not able to compete for HL binding in the liver. The nature of the HL binding site is not known. Heparan sulphate proteoglycans (HSPGs) may be involved [10,12,13] and the LDL-receptor-related protein may play a role in the degradation of HL [13]. However, in view of the specificity of HL binding, the HL binding site should have additional selective properties.

HL is heparin-releasable; the enzyme is found in the plasma after heparin injection or in perfusates after heparin-perfusion of the liver. We found that its binding site is also heparin-sensitive. Upon perfusion with heparin the HL binding capacity of rat liver is largely abolished [11]. This indicates either that the HL binding site is inactivated or that it is, like HL itself, released by heparin. In the present study we addressed this issue. We showed that heparin-containing perfusates of rat liver contain HL binding activity, which is probably exerted by a 73 kDa protein.

MATERIALS AND METHODS

Animals

Male Wistar rats (200–250 g) housed under controlled conditions (temperature 20–22 °C, light period from 7.00–19.00 h) with free access to Purina rat chow and water.

Isolation of HL

HL was isolated from heparin-containing rat liver perfusates as described previously [14]. HL triacylglycerol hydrolase activity was measured using an artificial glycerol-[9,10(*n*-^{14}C)]-trioleate (Amersham, Little Chalfont, Bucks., U.K.) emulsion in gum acacia, pH 8.5 [1]. Purified HL (showing one band on SDS/PAGE) was labelled with ^{125}I (Amersham) using lactoperoxidase and glucose oxidase (both from Boehringer-Mannheim, Mannheim, Germany) as described for LPL [15]. After iodination, the enzyme was separated from free iodine using a heparin-Sepharose column. The specific activity was in the range of 200–500 d.p.m./ng. Iodinated HL was stored at −80 °C until use.

Heparin-Sepharose chromatography

Heparin-containing perfusates from 2–3 rats (50 ml/rat) were applied on to a heparin-Sepharose column. After extensive washing with 10 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl and 10 % (v/v) glycerol, the column was eluted with a 60 ml linear salt gradient (0.2–1.0 M NaCl). Fractions (1 ml) were collected and tested for HL binding capacity by slot-blot assay as described below.

In some experiments the column was eluted batch-wise with 10 mM phosphate buffer, pH 7.0, containing 0.5 M NaCl and subsequently with the same buffer containing 1 M NaCl. The two fractions were collected, dialysed against 5 mM NH_4HCO_3 and freeze dried. After resuspension in 0.2–1 ml of PBS the fractions (which were designated the 0.5 M NaCl fraction and the 1 M NaCl fraction) were stored at −80 °C until use.

HL binding assays

Heparin-containing perfusate and fractions (1 ml), eluted with a linear salt gradient from heparin-Sepharose as described above,

Abbreviations used: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; HDL, high-density lipoprotein; HL, hepatic lipase; HSPG, heparan sulphate proteoglycan; IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase.

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were spotted on to nitrocellulose and the blot was blocked with 3% (w/v) BSA (Sigma, St. Louis, MO, U.S.A.). Subsequently the blot was incubated with ^{125}I-labelled HL (3 μg/ml) in 10 mM Tris/HCl, pH 8.0, containing 50 mM NaCl, 2 mM $CaCl_2$ and 1% (w/v) BSA. The blot was washed three times with the same buffer but without BSA and was scanned using a densitometer (Scanjet II CX; Hewlett Packard, Meriden, CT, U.S.A.).

For the solid phase binding assay, the wells of enzyme immunoassay (EIA)/RIA plates (Costar, Cambridge, MA, U.S.A.) were coated with 10–100 ng of the 0.5 M NaCl fraction (see the heparin-Sepharose chromatography section), with BSA (10–100 ng protein) or left uncoated (3 h at room temperature). After being washed four times with 200 μl of PBS/well, the wells were blocked overnight at 4 °C with 3% (w/v) BSA in PBS. Subsequently, the wells were incubated with different amounts of HL for 2 h at room temperature. The wells were washed four times with 200 μl PBS/0.1% (v/v) Tween 20 and thereafter incubated with a mix of monoclonal antibodies against rat HL [16] (1:5, 100 μl/well) for 2 h at room temperature. After being washed four times with 200 μl PBS/0.1% (v/v) Tween 20 per well, goat anti-mouse IgGs conjugated with alkaline phosphatase (TAGO Inc., Burlingame, CA, U.S.A.; 1:500, 100 μl/well) were bound for 2 h at room temperature. 4-Nitrophenyl phosphate (disodium salt) was used as a substrate and the absorbance was measured at 405 nm using a Thermomax microplate reader (Sopar Biochem, Nieuwegein, The Netherlands).

Cross-linking experiments

Cross-linking experiments were performed with the 0.5 M NaCl fraction by incubation with ^{125}I-labelled HL in the presence of the covalent linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC; Sigma) as described previously [17]. The sfraction was incubated with ^{125}I-labelled HL (3 μg/ml) in the presence of 11 mM EDAC for 20 min at 4 °C in 20 mM Hepes, pH 7.4, containing 128 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1 mM $CaCl_2$. Radioactively labelled proteins were separated by SDS/PAGE (7.5% gels) followed by autoradiography.

RESULTS

Heparin-releasability of HL binding activity

Upon perfusion with heparin-containing media, over 90% of HL activity is released into the perfusate. However, although the liver is depleted of HL, subsequent perfusion with HL-containing medium does not lead to rebinding of the enzyme. Therefore heparin either destroys the HL binding site or the binding site is heparin-releasable. To investigate the latter, heparin-containing perfusates were tested for HL binding capacity in a slot-blot assay. The perfusate was transferred on to nitrocellulose paper and incubated with ^{125}I-labelled HL. This resulted in a high binding capacity which was concentration dependent (Figure 1, bands 1 and 2). To test whether the ^{125}I-labelled HL binding represented binding to heparin still present in the perfusate, 10 μl of 100-fold concentrated heparin perfusate from which heparin had been removed by ultrafiltration was applied. Binding to this perfusate was as high as binding to the untreated heparin perfusate (Figure 1, band 3). Moreover, no binding was observed when ^{125}I-labelled HL was incubated with only Berry medium (140 mM NaCl/5.4 mM KCl/8.18 mM $MgSO_4$/0.8 mM Na_2HPO_4/25 mM $NaHCO_3$/2.54 mM $CaCl_2$/6 mM D-glucose) containing heparin (Figure 1, band 4). The binding of ^{125}I-labelled HL appeared to be specific since the signal on the autoradiogram had almost disappeared when the same experiment was performed in the presence of an excess of unlabelled HL (Figure 1, right panel).

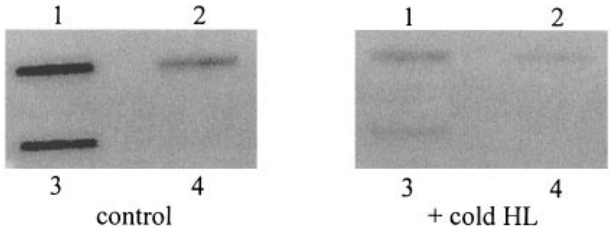

Figure 1 HL binding activity in heparin perfusate

Rat liver heparin perfusate was applied on nitrocellulose and incubated with ^{125}I-labelled HL as described in the Material and Methods section. The left panel shows binding of ^{125}I-labelled HL: lane 1, perfusate (1 ml); lane2, perfusate (0.5 ml); lane 3, concentrated perfusate (10 μl) after removal of heparin; and lane 4, heparin-containing Berry medium (1 ml), which had not passed through the liver. The right panel shows the same fractions but after incubation with ^{125}I-labelled HL in the presence of an excess of unlabelled (+cold) HL.

Heparin-Sepharose chromatography

Since the heparin-releasability of the HL binding activity suggests the involvement of heparan sulphates in the binding of the HL binding site, we investigated whether HL binding activity could be retained on a heparin-Sepharose column. Therefore heparin-containing liver perfusates from 2–3 rats were loaded on to a heparin-Sepharose column and eluted with a linear 0.2–1.0 M NaCl gradient. Fractions (1 ml) were collected and tested for HL binding capacity in a slot-blot assay. About 75% of the HL binding activity was retained on the column and binding activity was eluted in two major fractions at 0.3 M and 0.8 M NaCl (Figure 2). The HL binding capacity of the 0.8 M fraction coincided with the elution of HL activity (Figure 2).

Further characterization of HL binding in the perfusate was carried out with fractions obtained after stepwise elution from

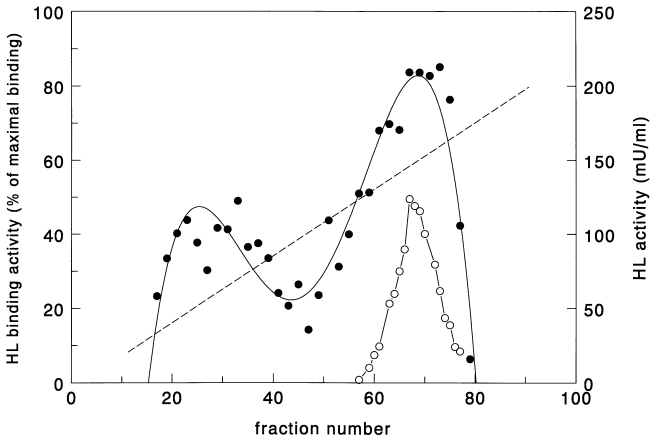

Figure 2 HL binding activity in heparin-Sepharose eluate

Heparin-containing rat liver perfusates were loaded on to a heparin-Sepharose column and eluted with a linear NaCl gradient (0.2–1.0 M). Fractions were collected and tested for HL binding activity using a slot-blot assay and for HL enzyme activity. About 75% of total binding activity was retained on the column. Binding activity (●) is expressed as a percentage of the maximal binding observed and HL enzyme activity (○) is expressed as m-units/ml. The results are the means of three experiments.

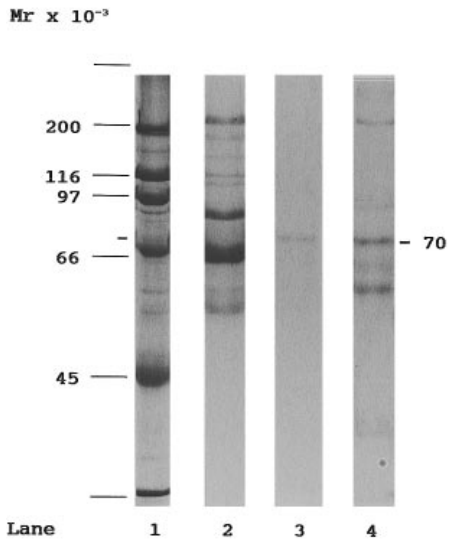

Figure 3 Proteins present in heparin-Sepharose eluates

Heparin-containing rat liver perfusates were loaded on to a heparin-Sepharose column and were eluted with 0.5 and 1.0 M NaCl. Fractions were collected and concentrated as described in the Materials and Methods section. Samples of the fractions (10 μl) were separated by SDS/PAGE (7.5% gels). Lane 2, whole perfusate before heparin-Sepharose chromatography; lane 3, 0.5 M NaCl fraction; lane 4, 1.0 M NaCl fraction and lane 1, molecular mass markers.

the heparin-Sepharose column with 0.5 M and 1.0 M NaCl respectively. The protein composition of both fractions was analysed by SDS/PAGE (Figure 3). The 0.5 M fraction was found to contain one major protein band of 70 kDa. The 1 M fraction contained two major protein bands of the same intensity of respectively 70 kDa and 57 kDa. The 57 kDa band represents HL, confirmed by Western blotting (results not shown). HL binding of the 0.5 M fraction was further studied using a solid-phase binding assay. The 0.5 M fraction was coated on to a 96-well plate and incubated with HL as described in the Materials and methods section. HL binding to the fraction was saturable; saturation was reached at concentrations greater than 10 m-units/ml, corresponding to 625 ng/ml (Figure 4). As a control, wells were first coated with BSA and then incubated with HL. No significant HL binding was found in these wells. These results indicate that the 0.5 M fraction contains a high-affinity HL binding factor.

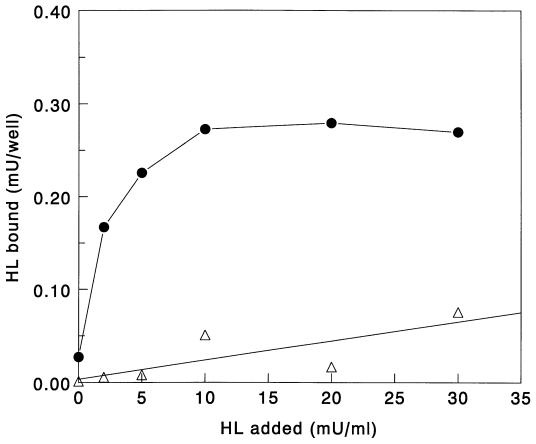

Figure 4 HL binding to the 0.5 M NaCl eluate after heparin-Sepharose chromatography

The 0.5 M NaCl fraction (see Figure 3, lane 3) was tested for HL binding capacity in a solid phase binding assay (●). Binding components were coated on to a microtitre plate and incubated with HL, monoclonal antibodies against HL were then bound followed by incubation with goat anti-mouse antibodies conjugated with alkaline phosphatase. 4-Nitrophenyl phosphate (disodium salt) was used as a substrate and the absorbance was measured at 405 nm. As a control, the wells were coated with BSA and tested for HL binding (△).

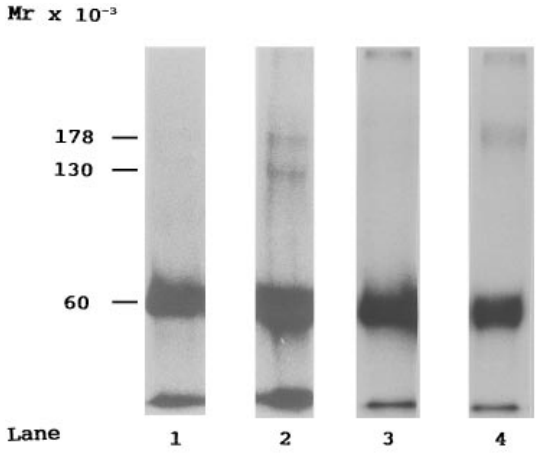

Figure 5 Cross-linking of the 0.5 M NaCl fraction with ^{125}I-labelled HL using EDAC

^{125}I-labelled HL was incubated with a sample of the 0.5 M NaCl eluate from a heparin-Sepharose column (see Figure 3) and without (lane 1) or with (lane 2) the covalent linker EDAC for 20 min as described in the Material and Methods section. Incubation of ^{125}I-labelled HL with (lane 4) or without (lane 3) EDAC in the absence of the 0.5 M NaCl fraction.

Identification of the HL binding protein

To study the involvement of the 70 kDa protein in HL binding we performed ligand blotting with the 0.5 M NaCl fraction. The fraction was subjected to reducing and non-reducing SDS/PAGE and blotted on to nitrocellulose membranes. The membranes were incubated with ^{125}I-labelled HL, and no binding was observed (results not shown). Since SDS/PAGE may have altered the HL binding activity, we studied the interaction of HL with components of the fractions containing HL binding activity by cross-linking ^{125}I-labelled HL to the native unmodified fraction. Upon addition of the cross-linker EDAC to the 0.5 M NaCl fraction in the presence of ^{125}I-labelled HL, ^{125}I-labelled complexes of 130 and 178 kDa were formed (Figure 5, lane 2). The major labelled band of 57 kDa represents free ^{125}I-labelled HL. In the absence of EDAC, only free ^{125}I-labelled HL was visible on the autoradiogram (Figure 5, lane 1). When ^{125}I-labelled HL was cross-linked with EDAC a 178 kDa complex was formed (Figure 5, lane 4).

DISCUSSION

HL is specifically bound to rat liver parenchymal cells [2] but until now no HL binding site has been identified.We have found that when rat livers are perfused with HL-containing medium no HL can be bound, indicating that all HL binding sites are occupied, whereas LPL can still bind to the liver [11]. Furthermore, the largest part of HL activity was bound to a heparin-sensitive binding site as most binding capacity was lost upon

heparin perfusion [11]. Moreover, after treatment of rats with adrenocorticotrophic hormone, HL activity in the liver was decreased and the number of heparin-sensitive HL binding sites had diminished, suggesting that regulation of HL activity in rat liver may occur at the level of the HL binding sites [11]. In the present study, we investigated the nature of the heparin-sensitive binding site. This binding site may be either destroyed or released by heparin. The latter hypothesis was confirmed by slot-blot experiments, where heparin-containing perfusates from rat liver were incubated with ^{125}I-labelled HL. A high binding capacity was observed, which was displaced by an excess of unlabelled HL, indicating that heparin-treated rat liver perfusates contained specific HL binding components. The high specific binding was not due to heparin present in the perfusate, because binding to perfusate from which heparin had been removed by dialysis was the same as that of untreated heparin perfusate. Moreover, heparin-containing medium which had not passed through the liver did not bind HL, providing additional evidence that the perfusate contains a specific HL binding activity, which cannot be attributed to the heparin in the medium. Therefore it can be concluded that the heparin-sensitive binding site of HL is probably heparin-releasable. However, we were not able to rule out the possibility that part of the heparin-induced reduction of HL binding might be due to inactivation of binding sites.

Release of the HL binding site with heparin indicates competition of heparin with HSPGs at the cell surface. There is some evidence that HSPGs are involved in HL binding [10,12,13]. Heparinase treatment of hepatoma cells overexpressing HL abolished HL-mediated binding and uptake of chylomicrons and very-low-density lipoproteins [12]. Also, Kounnas et al. [13] showed that proteoglycan-deficient Chinese hamster ovary cells were unable to internalize and degrade HL. However, for HL binding the HSPG should be specific, as LPL, which is structurally very similar and also binds to HSPGs, was not able to compete for HL binding. The specificity of HSPGs for the binding of HL was also suggested by Lookene et al. [18], who did not find any binding of HL to heparan-sulphate-covered sensor chips of a BIAcore system, whereas LPL was bound with a high affinity. It was suggested that this may be due to the source of the heparan sulphates used (endothelial), as hepatic heparan sulphates contain more heparin-like regions [19]. However, it is tempting to speculate that the lack of binding may have been due to the absence of a heparin-releasable HL binding component. Most heparin-releasable proteins bind to heparin-Sepharose, although with variable affinity. It appeared that HL binding activity in the heparin perfusates was also retained on a heparin-Sepharose column. Binding activity eluted in two peaks reaching a maximum at 0.3 M and 0.8 M NaCl. It is, therefore, possible that there are two different binding proteins with different affinities for heparin. However, HL also elutes at an NaCl concentration of 0.8 M and the peak of HL activity coincided with the elution of HL binding activity. It seems likely, therefore, that the second peak represents HL, which eluted together with its binding site.

When we analysed the proteins in the heparin-Sepharose eluate, the major protein present in the first peak (0.5 M NaCl eluate) had an apparent molecular mass of about 70 kDa. In the 1 M NaCl fraction this protein was also one of the major bands and it was of similar intensity to the 57 kDa band representing HL. The 70 kDa protein, therefore, appeared to be a good candidate for the HL binding protein. It is not likely that the 70 kDa protein is albumin, since BSA bound very little HL. Besides, the 70 kDa band in the total perfusate was clearly discernable from the albumin band of the marker proteins and also from the 66 kDa band, which probably represented rat albumin. Moreover, HL binding was not inhibited by anti-albumin antibodies (results not shown).

To establish the molecular mass of the HL binding component we performed ligand blotting experiments. However, after SDS/PAGE of a heparin-containing liver perfusate, using both reducing and non-reducing conditions, and subsequent blotting, no HL was bound. Because the presence of SDS could have changed the conformation of the binding protein to such an extent that binding of HL was no longer possible, we used another approach to establish the molecular mass of the binding protein. Cross-linking experiments were performed with the 0.5 M NaCl eluates, because no endogenous HL was present in this fraction, and binding of HL to this fraction was found to be saturable using solid-phase HL binding assays. Incubation of this fraction, which contained the HL binding component in its native form, with ^{125}I-labelled HL in the presence of a cross-linker resulted in the formation of two complexes, one of 130 kDa and one of 178 kDa. The latter complex was also formed when ^{125}I-labelled HL was cross-linked with EDAC in the absence of the 0.5 M NaCl eluate, and probably represents a multimer of HL. This means that HL (which has a molecular mass of 57 kDa) was crosslinked to a protein of about 73 kDa. Our results show that a heparin-releasable HL binding protein of about 73 kDa is present in rat liver. The heparin-releasable HL binding component can be bound to heparin. Therefore, *in vivo*, this binding protein probably associates with proteoglycans on the parenchymal cell surface. The binding protein itself is not likely to be a proteoglycan, since these proteoglycans usually smear on SDS/PAGE. It is also known that proteoglycans do not stain very well with Coomassie Blue [20], and this was not the case for the HL binding protein. However, we cannot totally exclude the possibility that the binding protein is a proteoglycan.

Sivaram et al. [21] found that LPL also associated with a heparin-releasable binding protein. However, this protein had an apparent molecular mass of 116 kDa and was found in endothelial cells. As HL is not present in endothelial cells, and as there is no competition between LPL and HL for binding, this binding protein is probably not involved in the binding of HL.

We are currently investigating the properties of the heparin-releasable HL binding protein.

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