Inactive hepatic lipase in rat plasma

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Abstract  Hepatic lipase activity is detectable in liver but also in adrenal glands, ovaries, and plasma. The subunit size of hepatic lipase in liver, adrenal glands, and nonheparin plasma was compared. Hepatic lipase in liver and adrenal glands appeared as a 55 kDa band. In liver, a faint band of lower size was also detected. In nonheparin plasma, hepatic lipase appeared as a doublet of 57 kDa and 59 kDa. When activity/mass ratio was calculated, similar values were obtained for liver and adrenal glands. In plasma this value was much lower. After heparin administration in vivo, hepatic lipase activity in plasma increased nearly 100-fold with appearance of an additional 55 kDa band in postheparin plasma. This band coeluted with activity after preparative polyacrylamide gel electrophoresis. Differences in size persisted after digestion with peptide-N-glycosidase F. A progressive increase in 57 kDa and 59 kDa in postheparin plasma followed disappearance of the 55 kDa band, suggesting that these larger bands originate from the smaller form. In plasma, both smaller and larger forms were associated with HDL, but not with LDL or VLDL. We conclude that rat plasma contains a larger form of hepatic lipase that is inactive in vitro assay.—Galan, X., J. Peinado-Onsurbe, J. Julve, D. Ricart-Jané, M. Q. Robert, M. Llobera, and I. Ramírez. Inactive hepatic lipase in rat plasma. J. Lipid Res. 2003. 44: 2250–2256.

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Hepatic lipase is involved in the metabolism of remnant lipoproteins (1) and triglyceride-rich high-density lipoproteins-2 (2). As a result of triglyceride and phospholipid hydrolysis, hepatic lipase promotes the uptake of HDL cholesterol by the liver (3), and generates preβ1 HDL (4). Although mouse strains with targeted inactivation of the hepatic lipase gene show only mild dyslipemia (5), kinetic studies in hepatic lipase-deficient mice provide in vivo evidence of the significant role of hepatic lipase in selective delivery of HDL-cholesteryl esters to the liver (6). Studies in double knock-out mice lacking both apolipoprotein E (apoE) and hepatic lipase also support a role for this enzyme in the selective delivery of cholesterol to steroidogenic tissues and the liver (7, 8). The relevance of hepatic lipase in lipoprotein metabolism is supported by the finding that total plasma cholesterol levels are decreased in transgenic rabbits overexpressing human hepatic lipase compared with nontransgenic littermates (9).

Hepatic lipase was described first in postheparin plasma as a liver-derived enzyme (10), but hepatic lipase is also found in adrenal glands and ovaries (11, 12). Synthesis of this enzyme in adrenal glands was observed only in newborn animals (13), but not in adults (14). Hepatic lipase mRNA in hepatocytes encodes a 55–59 kDa protein secreted to the extracellular space (14–17). In an early study on hepatic lipase synthesis in adrenal glands, Doolittle et al. (14) reported that subunit size was similar in adrenal glands and in liver. However, in a more recent report, Schoonderwoerd et al. (18) concluded that subunit size of hepatic lipase is lower in adrenal glands than in liver.

Heparin administration releases a large amount of hepatic lipase into the blood stream (10, 19), but hepatic lipase activity is also detectable in nonheparin plasma (20, 21). Perfused liver continuously releases hepatic lipase, and so activity in plasma correlates with hepatic activity (17). It was suggested that hepatic lipase in adrenal glands may originate in liver (14). Not much is known about hepatic lipase in plasma, although it may have relevant biological functions. We report here some unique properties of hepatic lipase in rat plasma.

MATERIALS AND METHODS

Animals

Wistar rats were obtained from our own colony, and were killed by decapitation. Blood was collected in vials containing EDTA and plasma was obtained by centrifugation (30 min at 10,000 g at 4°C). The liver and adrenal glands were immediately

Abbreviations: LDP, lipoprotein-depleted plasma; N-PAGE, native PAGE; PNG, peptide-N-glycosidase F.

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frozen in liquid N2. Tissue homogenates were prepared in 10 mM HEPES (pH 7.5) (supplemented with 1 mM EDTA, 1 mM dithiothreitol, 10 mM/m/ ml aprotonin, 25 mM benzamidine, 1 µM leupeptin, 1 µM pepstatin, 0.2 mM PMSF, 5 U/ml heparin, and 1.5% Triton X-100) in a polytron homogenizer (Kinematica GmbH, Luzern, Switzerland). Homogenates were clarified by centrifugation (10 min at 10,000 g at 4°C). Clarified supernatants and blood plasma were kept at −40°C until determinations of hepatic lipase activity.

Postheparin plasma was obtained from animals injected with heparin (20 U/kg, i.v.) 15 min before they were killed. When the time course was studied, blood was obtained from the tail at indicated times. Heparin-released hepatic lipase from perfused rat liver was obtained as described previously (17).

All experimental procedures were approved by the Committee on Animal Care of the University of Barcelona.

**Hepatic lipase activity**

Hepatic lipase activity was determined following Ehnholm et al. (22) as described elsewhere (25). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of oleate per min at 25°C.

**Western blot analysis of hepatic lipase**

Samples were electrophoresed in 10% or 7.5% polyacrylamide gels (SDS-PAGE) (24). The separated proteins were then transferred to Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting. Thereafter, the membranes were soaked in blocking solution (2% BSA in PBS) for 90 min at 37°C, rinsed (5 × 5 min in 100 ml of rinsing solution: 1% SDS, 1% Triton X-100, 0.5% defatted powdered-milk in PBS), and incubated overnight at 4°C with the primary antibody [rabbit anti-rat hepatic lipase serum (17) diluted 1:1,000 in buffer A (0.1% SDS, 0.1% Triton X-100, 0.5% defatted powdered-milk in PBS) supplemented with 5% BSA and 0.5% gelatin]. Then, membranes were rinsed as indicated above and incubated for 30 min at room temperature with the secondary antibody [biotin-labeled goat anti-rabbit IgG (Vector, Burlingame, CA) diluted 1:5,000 in buffer A]. After rinsing as described above, the membranes were incubated with the ABC-complex [VECTASTAIN (ABC-kit), Vector] and developed with the ECL-system (Amersham, Little Chalfont, United Kingdom). Films were analyzed with Phoretix 1D Gel Analysis software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) after scanning in an EPSON GT-8500. In each gel, a lane was loaded with a constant amount of hepatic lipase to correct for differences in the results.

**Heparin-Sepharose affinity**

Twenty-five µl of either nonheparin or postheparin plasma was added to 0.5 ml of heparin-Sepharose (Amersham-BioScience) and incubated for 60 min at 4°C. Unbound proteins were obtained by centrifugation. The gel was then rinsed five times with five-bed volumes of 10 mM Tris-HCl (pH 8.2) containing 0.15 M NaCl. Bound proteins were recovered by incubating the gel for 60 min at 4°C with Tris buffer containing 1.2 M NaCl. Identical amounts of protein from whole plasma (WP) and from unbound and bound fractions were precipitated with 3.5% perchloric acid and analyzed by Western blotting as indicated above.

**Native electrophoresis**

Preparative native PAGE (N-PAGE) was performed in Model 491 PrepCell (Bio-Rad, Hercules, CA) following the manufacturer’s instructions. Nonheparin or postheparin plasma (1.5 ml, diluted 4:1 with 40% sucrose, 0.01% bromophenol blue) was loaded onto 5 cm long 4% polyacrylamide gel and run in 90 mM Tris, 80 mM boric acid, 3 mM EDTA (pH 8.35), for 24 h at 5 W and 4°C. Fractions were collected (5 ml) at a flow rate of 0.25 ml/min. An aliquot of each collected fraction was used to determine hepatic lipase activity; another aliquot was used for Western blot analysis after SDS-PAGE.

Analytical N-PAGE was performed essentially as indicated above in MiniProtein II (Bio-Rad). After electrophoresis, analytical gels were stained with Coomassie blue.

**Digestion with peptide-N-glycosidase F**

Samples were precipitated with trichloroacetic acid (50%) and resuspended in 0.25 M Na2HPO4 (pH 7.4), 50 mM EDTA, 2% octylglucoside, 0.2% SDS, and 2 mM PMSF, containing 1 U/ml of N-glycosidase F (Boehringer, Mannheim). Samples were boiled for 1 min and then incubated for 24 h at 37°C. Proteins were then resolved in 7.5% SDS-PAGE, transferred to nylon membranes, and hepatic lipase was detected by Western blot as indicated above.

Plasma lipoproteins were isolated by sequential centrifugation (25).

**RESULTS**

First, effects of several physiological conditions on hepatic lipase activity in liver, adrenal glands, and plasma were examined. Hepatic lipase activity in liver was significantly lower in newborn (1 day old) and lactating (15 days old) rats than in adults (104 ± 8, 94 ± 14, and 522 ± 21 mU/g respectively). In newborn and lactating animals, hepatic lipase activity in plasma (1.6 ± 0.5 and 0.9 ± 0.4 mU/ml, respectively) and in adrenal glands (12 ± 10 and 23 ± 15 mU/g, respectively) was also significantly lower than the corresponding adult values (plasma: 4.7 ± 0.7 mU/ml; adrenal glands: 389 ± 18 mU/g). In adult animals, 48 h fasting significantly decreased hepatic lipase in liver (311 ± 45 mU/g), adrenal glands (313 ± 23 mU/g), and plasma (2.6 ± 0.2 mU/ml). Catecholamines are involved in the effect of fasting on hepatic lipase in liver (26). One hour after adrenaline administration [as in (26)], hepatic lipase in liver and adrenal glands decreased by 25% (378 ± 17 and 304 ± 11 mU/g respectively); in plasma, hepatic lipase was halved (2.1 ± 0.1 mU/ml). There was a highly significant correlation (P < 0.001) between hepatic lipase activity in liver and adrenal glands for all situations described above (Fig. 1).

It was reported previously that both subunit size and functional molecular mass of hepatic lipase in adrenal glands differ from corresponding values in liver (18). Therefore, we compared the subunit size of hepatic lipase in liver, adrenal glands, and nonheparin plasma (Fig. 2A). In this experiment, a heart sample was run as a negative control. The size of the hepatic lipase subunit in adrenal glands was identical to that in liver (55 kDa). In the liver, a faint band of lower size was also detected. Since hepatic lipase activity was measured in these samples, the ratio between activity (mU/ml) and the densitometric value of the hepatic lipase band (arbitrary densitometric U/ml: ADU/µl) was calculated. This activity to mass ratio was 105 mU/ADU and 127 mU/ADU for adrenal and liver hepatic lipase, respectively. In nonheparin plasma, a larger size band (57 kDa) was observed unexpectedly whose ac-
Activity to mass ratio was only 0.53 mU/ADU, indicating the presence of a substantial quantity of inactive mass. Therefore, this very inactive form of hepatic lipase was investigated further.

Next, hepatic lipase in nonheparin and postheparin plasma was compared. Fifteen min after intravenous heparin administration, hepatic lipase activity in plasma was 160 ± 13 mU/ml, almost 100-fold higher than before heparin administration (2 ± 1 mU/ml). When nonheparin and postheparin plasma samples were electrophoresed in 10% SDS-PAGE, hepatic lipase in postheparin plasma appeared as a broader band of smaller mass, compared with the nonheparin plasma sample (Fig. 2B). When the same samples were electrophoresed in 7.5% SDS-PAGE, hepatic lipase in nonheparin plasma appeared as two separate bands of 57 kDa and 59 kDa. Postheparin plasma hepatic lipase appeared as a major band of 55 kDa, and two smaller bands of 57 kDa and 59 kDa.

To separate larger and smaller forms of hepatic lipase, postheparin plasma was loaded onto preparative N-PAGE. Postheparin plasma hepatic lipase activity eluted from N-PAGE as a defined peak collected in the first 15 fractions (Fig. 3); these fractions contained the 55 kDa band. In later fractions, the higher Mr bands were observed. When nonheparin plasma was loaded onto NPAGE, neither an activity peak nor the 55 kDa band was detected. Both 57 and 59 kDa bands appeared in similar fraction numbers to those collected from postheparin plasma.

To test whether hepatic lipase in nonheparin plasma had reduced affinity for heparin, nonheparin and postheparin plasma samples were incubated with heparin-Sepharose. Unbound and bound proteins were obtained in single fractions. Both 57 kDa and 59 kDa bands from nonheparin plasma were recovered in the bound fraction (Fig. 4), whereas the unbound fraction did not contain hepatic lipase of either size. When postheparin plasma was applied to heparin-Sepharose, all bands (55 kDa, 57 kDa, and 59 kDa) were recovered in the bound fraction. These results are consistent with the high recovery of activity in the bound fraction (95–100% of the nonheparin and postheparin plasma hepatic lipase activity; data not shown).

Next, nonheparin and postheparin plasma samples and heparin-perfusate of rat liver were incubated with peptide-N-glycosidase F (PNG) to remove oligosaccharide chains (Fig. 5). After PNG hydrolysis, hepatic lipase in nonheparin plasma samples and heparin-perfusate of rat liver were incubated with peptide-N-glycosidase F (PNG) to remove oligosaccharide chains.
arin plasma appeared as a major band of 49 kDa; a faint band of 45 kDa was also observed. Hepatic lipase in postheparin plasma appeared as a doublet of 49 kDa and 45 kDa after PNG digestion. In the heparin perfusate of rat liver, hepatic lipase appeared as a 55 kDa band. After PNG hydrolysis, a major band of 45 kDa and a minor band of 49 kDa were detected.

To study the time course for appearance of the 55 kDa band in postheparin plasma, plasma samples were obtained at various time points after intravenous heparin injection. In these samples, hepatic lipase activity was determined (Fig. 6, upper panel), and different Mr bands were quantified by Western blot after 7.5% SDS-PAGE (Fig. 6, middle and lower panels). Hepatic lipase activity in plasma (and the 55 kDa band) increased rapidly after heparin administration and decreased thereafter. The sum of 57 kDa and 59 kDa bands remained constant in control animals, but increased progressively in heparin-injected rats. Differences became significant 2 h after injection.

To study the distribution of hepatic lipase in plasma lipoproteins, lipoprotein fractions were obtained by sequential ultracentrifugation. Lipoproteins were characterized by their triglyceride and cholesterol content, mobility on 4% N-PAGE, and apolipoprotein profile on 7.5% SDS-PAGE (data not shown). The presence of hepatic lipase in each lipoprotein fraction, as well as in WP and lipoprotein-depleted plasma (LDP), was determined by Western blot (Fig. 7). Neither VLDL nor LDL fractions contained hepatic lipase. Hepatic lipase was associated with both nonheparin and postheparin HDL. Hepatic lipase was detected in both nonheparin and postheparin LDP.

**DISCUSSION**

**Hepatic lipase in liver and adrenal glands**

The physiological function of hepatic lipase in cholesterol delivery to the liver and steroidogenic tissues has gained some clarity in recent years. However, the origin of hepatic lipase in adrenal glands (paradigm of steroidogenic tissue) remains obscure. A hepatic origin is supported by the early studies of Doolittle and coworkers (14), who failed to detect hepatic lipase synthesis in adrenal glands from adult animals. In this scenario, the presence of hepatic lipase in plasma is of physiological relevance. However, no studies have focused on plasma hepatic lipase. Our results showing that i) a variety of physiological conditions affected hepatic lipase activity in plasma and adrenal glands similarly as in liver, and ii) there is a highly significant correlation between hepatic lipase activity in liver and adrenal glands, support a hepatic origin for adrenal gland hepatic lipase. Therefore, a relevant role for this enzyme in plasma is suggested since hepatic lipase activity in adrenal glands will depend on transport in the bloodstream from liver.

Synthesis and secretion of hepatic lipase in liver cells has been studied in both hepatoma cells in culture (15, 16, 27) and native hepatocytes (28, 29, 17). It is well established that these cells continuously secrete fully active 55–
59 kDa hepatic lipase. In addition, hepatic lipase exists as a partially glycosylated form of lower subunit size (52–55 kDa) inside cells. In whole liver extracts, the 55 kDa form of hepatic lipase was detected, but the 52 kDa form was not clearly distinguishable, in contrast to the previous observation with isolated hepatocytes. In these cells, the 52 kDa form accounts for about 30% of total hepatic lipase (17). The discrepancy between results using whole tissue and parenchymal cell extracts is explained by the accumulation of mature (55 kDa) hepatic lipase molecules in the Space of Disse (30).

In adrenal glands the 55 kDa form of hepatic lipase was detected, and the ratio between activity and Western blot-based mass units was similar to that in liver. In contrast, the functional molecular mass of hepatic lipase was reported to be higher with a lower subunit size in adrenals compared with liver (18). Western blot analysis was used for estimations of subunit size in our experiments and in the results of Schoonderwoerd et al. (18). Doolittle and co-workers (14) also performed Western blot and concluded that the subunit size of hepatic lipase in adrenal glands is identical to that of the hepatic enzyme. In all of these studies, the antibody used was a rabbit antiserum to purify rat liver hepatic lipase. Therefore, there is no readily apparent explanation for this discrepancy in estimates of the subunit size for hepatic lipase in adrenal glands.

**Inactive hepatic lipase in rat (nonheparin) plasma**

Low but detectable hepatic lipase activity has been reported in rat (20, 31, 17) and human nonheparin plasma (21), although Mao, Rechtin, and Jackson (32) did not find immunoreactive hepatic lipase in human plasma. After heparin administration, hepatic lipase is released from binding sites in liver (19), adrenal glands, and ovaries (12), and accumulates in plasma (10). Indeed, we observed a huge increase in hepatic lipase activity in plasma after heparin administration. We suggest that this activity corresponds to the 55 kDa band detected in postheparin plasma, which is absent in nonheparin plasma. Note that the time course of the appearance of hepatic lipase activity in plasma after heparin injection matches that of the 55 kDa band. In addition, the activity profile obtained from postheparin plasma after preparative N-PAGE matches the presence of the 55 kDa band.

In addition to the transient appearance of the 55 kDa form of hepatic lipase in plasma after heparin administration, our results reveal a highly abundant form in both nonheparin and postheparin rat plasma, which is larger in size than liver and adrenal gland forms. We conclude that the doublet (57 kDa and 59 kDa) bands observed in both nonheparin and postheparin plasma after 7.5% SDS-PAGE correspond to hepatic lipase because i) they are recognized by antibodies to heparin lipase; ii) like the 55 kDa form, they bind to heparin-Sepharose and are eluted at high NaCl concentration; iii) PNG digestion decreased molecular size of the 57/59 kDa bands by 8–10 kDa, similar to the decrease in hepatic lipase obtained by heparin perfusion of isolated livers; and iv) these bands, as well as the 55 kDa band, are associated with LDL but not with HDL or VLDL. However, a definitive conclusion requires further studies with purified material and both peptide map and sequencing analysis.

These 57/59 kDa forms are inactive in the in vitro assay. The activity to mass ratio of hepatic lipase in whole nonheparin plasma is 1:240 of that in liver. A similar conclusion can be drawn from the comparison of activity and mass eluted from heparin-Sepharose gels. Catalytic activity is rapidly lost when hepatic lipase is free in solution unless the enzyme is bound to heparin (33, 17). This might explain lack of activity in nonheparin plasma. However, it is reasonable that inactivation may be a consequence of the modification of the subunit structure responsible for the increase in molecular size. Note that the decrease in both activity and the 55 kDa band after heparin injection occurs when the mass of the 57 kDa and 59 kDa bands increases. An alternative hypothesis is that reuptake and degradation of the active 55 kDa band is associated with the late appearance of high molecular size hepatic lipase from heparin-stimulated livers. Kinetic experiments using injected radioabeled hepatic lipase are required to determine whether the 55 kDa form is directly converted into 57/59 kDa forms.

Our preparations containing 57/59 kDa forms of hepatic lipase are not pure protein samples; activity was measured in WP, and in preparative N-PAGE and heparin-Sepharose fractions, which contain many proteins in addition to hepatic lipase. Therefore, the enzyme may not be inactive, but catalytic activity could be masked because of association with some plasma component. Very recently (34), it was reported that hepatic lipase activity is inhibited by association with several HDL fractions or pure apoA-I. Note that we found that hepatic lipase is associated with HDL in rat plasma. If this hypothesis is correct, some unique mechanism in the smaller form associates its appearance in plasma after heparin administration, with the increase in activity.

Concerning the mechanisms involved in the release of hepatic lipase to bloodstream, we can only make suggestions requiring further experimental study. Perfused livers spontaneously release active (55 kDa) hepatic lipase (17). Therefore, in the whole animal, the liver may spontaneously release hepatic lipase molecules that are then inactivated. Furthermore, this release from the liver may not be spontaneous. HDL displaces hepatic lipase from the cell surface in both HepG2 cells and CHO cells expressing human hepatic lipase (34). HDL may thus also release hepatic lipase from binding sites in liver into the bloodstream. We found a large amount of hepatic lipase associated with HDL, but this may have occurred after hepatic lipase molecules were released from binding sites. Notwithstanding whether the release is spontaneous or induced, hepatic lipase in nonheparin plasma is inactive (in the in vitro assay) and with an increase in size. Therefore, these processes may take place simultaneously with or immediately after the release. Although our results demonstrate that the difference in size is not due to a modification of the oligosaccharide moiety, we can draw no further conclusions about the nature of the size modification.
Is there a function for inactive hepatic lipase in nonheparin plasma?

Hepatic lipase and lipoprotein lipase belong to the same gene family (35, 36). The lipoprotein lipase gene is expressed in adipose tissue, in skeletal muscle and heart, and in many other extrahepatic tissues. Lipoprotein lipase can be released from binding sites in endothelial cells, and released enzyme molecules are rapidly and irreversibly inactivated through monomerization. In human plasma, a high amount of inactive lipoprotein lipase has been described (37); inactivation was suggested to result from monomerization. There was no difference in monomer size between nonheparin and postheparin plasma lipoprotein lipase, and both active and inactive lipoprotein lipase were associated with LDL and HDL (38). Therefore, lipoprotein lipase and hepatic lipase in nonheparin plasma, although not identical, share some similarities: a high abundance of an inactive form that it is associated with some plasma lipoproteins.

Inactive lipoprotein lipase in plasma is finally degraded in liver cells (39), but inactive lipoprotein lipase is also involved in the liver uptake of remnant lipoprotein (40). Is there any similar role for hepatic lipase in nonheparin plasma? Since both active and inactive hepatic lipase are associated with HDL, hepatic lipase may facilitate the uptake of these lipoprotein by adrenal glands and other steroidogenic tissues. In addition, as discussed above, adrenal glands contain active hepatic lipase that may originate in the liver. Are the larger forms of hepatic lipase involved in transport of hepatic lipase molecules from liver to adrenal glands where the enzyme can be reactivated? Although our results leave many questions unanswered, they shed some light on a feature of hepatic lipase biology that has not been addressed previously. As these questions are investigated, the role of hepatic lipase in plasma and in steroidogenic tissues will be better understood.

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