Lipolysis of very low density lipoproteins by heparan sulfate proteoglycan-bound lipoprotein lipase

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Abstract An in vitro assay to study lipolysis of very low density lipoproteins (VLDL) by heparan sulfate proteoglycan (HSPG-bound lipoprotein lipase (LPL) was developed. Optimal conditions for VLDL lipolysis by HSPG-bound LPL were obtained by incubating plastic wells with 0.5 μg HSPG and 1.5 μg LPL, subsequently. Control experiments with heparinase indicate that at least 90% of the LPL activity is derived from LPL bound to heparan sulfate chains. For HSPG-LPL-mediated lipolysis, the apparent K_m and V_{max} values were 0.36 ± 0.11 mM VLDL-triglycerides and 1.2 ± 0.1 μM free fatty acids/min·ng LPL, respectively. The mean intra-assay and inter-assay coefficients of variance were 5% and 8%, respectively.—**de Man, F. H. A. F., F. de Beer, A. van der Laarse, A. H. M. Smelt, and L. M. Havekes.** Lipolysis of very low density lipoproteins by heparan sulfate proteoglycan-bound lipoprotein lipase. *J. Lipid Res.* 1997. **38:** 2465–2472.

Supplementary key words triglyceride • VLDL metabolism

Lipoprotein lipase (LPL, EC 3.1.1.34) is the key enzyme involved in the hydrolysis of chylomicron and very low density lipoprotein (VLDL) triglycerides (1, 2). After synthesis in parenchymal cells, LPL is secreted and transported across the endothelium where it binds to heparan sulfate proteoglycans (HSPG) at the luminal surface of endothelial cells (3, 4). Although functional LPL acts in vivo as a proteoglycan-bound enzyme, its kinetics in vitro are commonly studied with LPL in solution. Only few studies with heparin-Sepharose immobilized LPL have been reported previously (5, 6). In these studies, a higher Michaelis-Menten constant (K_m) and lower maximum reaction velocity (V_{max}) were noted for heparin-Sepharose immobilized LPL as compared to LPL in solution. This observation was in accordance with in vivo studies, showing that heparin-induced release of LPL from the vessel wall resulted in rapid clearance of plasma triglycerides (7, 8), a phenomenon explained by increased accessibility of the enzyme for its substrate.

In addition to the observed difference in lipolysis rate, it is conceivable that the proximity of heparin or HSPG influences directly the substrate–enzyme interaction. As triglyceride-rich lipoproteins come into contact with the endothelium-bound LPL, the particle has to reside transiently at the lipolytic site. This interaction between the lipid particle and vessel wall components is considered to enhance the stability of the lipoprotein-LPL complex (9), a process probably mediated by apolipoproteins (apo). Specific binding sites for LPL have been reported on apoC-II and B-100 (10, 11), whereas apoE is known to bind to heparan sulfate chains (9, 12). Ji et al. (12) demonstrated that enrichment of β-VLDL with apoE enhanced the binding of the β-VLDL to liver cells 4- 5-fold, an effect which was suggested to be mediated by the interaction of apoE with HSPG. In addition, they also found that mutant apoE showed reduced affinity to isolated HSPG, a finding which was confirmed by others (13). It was speculated that a defective interaction between apoE and HSPG may be associated with an impaired lipolysis of triglyceride-rich lipoproteins by HSPG-bound LPL (14,15). However, this hypothesis could not be tested so far as a lipolysis assay with HSPG-bound LPL was not available. The present paper presents a novel, reproducible and rapid in vitro assay for lipolysis of VLDL using HSPG-bound LPL.

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; HLP, hyperlipoproteinemia; HSPG, heparan sulfate proteoglycan; K_m, Michaelis-Menten constant; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TG, triglyceride; Tris, Tris(hydroxymethyl)-aminomethane; VLDL, very low density lipoprotein; V_{max}, maximum velocity.

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MATERIALS AND METHODS

Lipids and lipoproteins

Venous blood from healthy, normolipidemic apoE3 homozygotes was collected after an overnight fast. Serum was obtained after centrifugation at 1500 *g* for 15 min at room temperature. VLDL was isolated by ultracentrifugation as described by Redgrave, Roberts, and West (16). Protein content of the VLDL samples was determined by the method of Lowry et al. (17). Triglyceride concentrations of the VLDL fraction were measured enzymatically using a test kit (Sigma Chemicals, St. Louis, MO). ApoE phenotyping was performed by isoelectric focusing according to Havekes et al. (18).

Lipoprotein lipase

LPL was purified from fresh bovine milk as described previously (19). The isolated fraction was resuspended in 20 mM NaH_2PO_4, 50% glycerol and stored in aliquots at −80°C. Isolated LPL was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 4–20%) (**Fig. 1**) (20). Proteins were stained with Coomassie Brilliant blue or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were incubated with monoclonal antibody 5D2 (prepared by Dr. J. D. Brunzell et al. (21), University of Washington, Seattle), and rabbit anti-mouse IgG conjugated to peroxidase (DAKO, Glostrup, Denmark) was used as second antibody. A clear band with the approximate molecular weight of bovine LPL (56 kDa) was detected, which accounted for 51% of the total amount of protein in the sample (Fig. 1, panel A). This band reacted with the 5D2 antibody to bovine LPL (Fig. 1, panel B). The specific activity of the isolated LPL was 10.9 U/mg.

Assay of lipolysis with heparan sulfate proteoglycan-bound lipoprotein lipase

HSPG, isolated from basement membrane of mouse sarcoma cells, were purchased from Sigma. The assay was performed in 96-well microtiter plates (Greiner GmbH, Frichenhausen, Germany). Wells were incubated with different amounts of HSPG (as indicated) for 18 h at 4°C, washed three times with phosphate-buffered saline (PBS) and subsequently blocked for 1 h at 37°C with PBS containing 1% (w/v) essentially free fatty acid (FFA)-free bovine serum albumin (BSA) (Sigma). Then, the wells were incubated with different amounts of LPL per well (as indicated), diluted in Tris-glycerol buffer (0.1 M Tris, 20% (v/v) glycerol, pH 8.5) for 1 h at 4°C. After washing the wells three times with Tris buffer (0.1 M Tris, pH 8.5), lipolysis was started by adding various amounts of VLDL (as indicated) to the preconditioned well in the presence of 1% (w/v) essentially FFA-free BSA and placing the plate in a shaking incubator at 37°C. The rate of FFA release proved to be linear in time up to 10 min. The reaction was stopped after 10 min by the addition of Triton X-100 (1% (v/v), final concentration), followed by vortexing and cooling on ice. FFA concentrations were measured in triplicate using a NEFA C kit (Wako Chemicals GmbH, Germany).

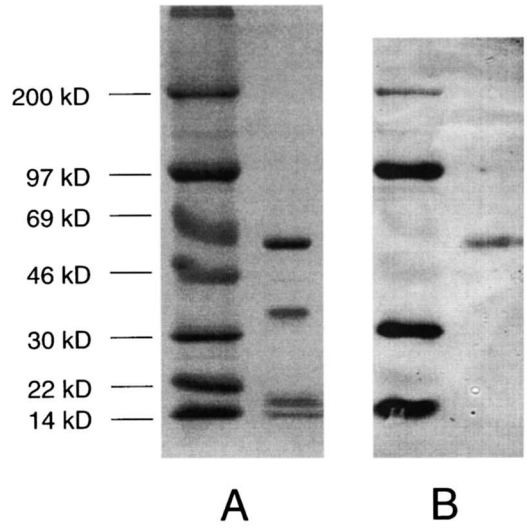

Fig. 1. Isolated bovine LPL was analyzed by SDS-PAGE gel electrophoresis (4–20%). Proteins were stained with Coomassie Brilliant blue (panel A) or transferred to nitrocellulose membranes. Blots were incubated with monoclonal antibody 5D2, and rabbit anti-mouse IgG conjugated to peroxidase was used as second antibody (panel B). Molecular weight standards are indicated in the left lanes of panel A and B.

As control, HSPG-LPL-coated wells were incubated with a mixture of 2.4 U heparinase I/ml (EC 4.2.2.7.; Sigma) and 2.4 U heparinase III/ml (EC 4.2.2.8.; Sigma) in PBS for 20 min at 37°C. The wells were washed with Tris buffer and lipolysis was performed as described above.

To check whether LPL would detach from the HSPG–complexes in the presence of VLDL, the following control experiment was performed. Wells were preconditioned as described above, with the exception that ^{125}I-labeled LPL was used. Then, the wells were incubated with 0.6 mM VLDL-TG or Tris buffer for 10 min at 37°C. After washing the wells three times with Tris buffer, the ^{125}I-labeled LPL bound to HSPG was dissolved in 0.2 N NaOH for quantitation.

Reproducibility was assessed by comparing the lipolysis of a VLDL sample freshly isolated from serum of a normolipidemic subject, and VLDL from the same subject but stored for 1 and 4 weeks, respectively. For storage, serum samples were brought to a final concentration of 10% (w/v) sucrose, 10 μM EDTA, capped under nitrogen, snap-frozen in liquid nitrogen, and stored at

−80°C. Under these conditions, lipoprotein size and biological properties have been shown to remain intact for months (22).

Assay of lipolysis with lipoprotein lipase in solution

The VLDL samples were diluted in 0.1 M Tris, 1% (w/v) essentially FFA-free BSA, pH 8.5. The incubation was started by adding 7 ng LPL per well, followed by vortexing and incubation at 37°C. The reaction was stopped by the addition of 1% (v/v) Triton X-100, 0.1 M Tris, vortexing, and cooling on ice. A blank sample was obtaining by adding Triton prior to the addition of LPL and maintenance on ice. FFA concentrations were determined in triplicate. The rate of FFA release by LPL was linear for at least 6 min, as used in this assay.

Labeling of very low density lipoproteins and lipoprotein lipase

VLDL was iodinated using the [^{125}I]-iodine monochloride method of Bilheimer, Eisenberg, and Levy (23). [^{125}I]-iodide (sp act 15.5 mCi/μg) was purchased from Amersham (Buckinghamshire, UK). After iodination, VLDL was dialyzed extensively at 4°C against PBS for 24 h and stabilized with 1% (w/v) BSA (fraction V, Sigma). Between the different ^{125}I-labeled VLDL samples the specific radioactivity ranged from 150 to 200 cpm/ng protein. The stabilized ^{125}I-labeled VLDL was stored at 4°C and used within two weeks.

LPL was iodinated using the IODO-BEADS® Iodination Reagent (Pierce, Rockford, IL). Free ^{125}I was removed by Sephadex G-25 gel filtration with 50 mM Tris, 1 M NaCl, 0.01% Tween-80 as the eluent. The specific activity of ^{125}I-labeled LPL was 300 cpm/ng protein. ^{125}I-labeled LPL was stabilized with 0.1% (w/v) essentially FFA-free BSA and stored at −20°C.

Binding assays

Binding of VLDL. Plastic wells (96-well microtiter plates) were coated with 0.5 μg HSPG per well and subsequently incubated with 1.5 μg LPL per well, exactly as described above. After washing the plates two times with ice-cold PBS, the binding of VLDL to HSPG-bound LPL was determined by incubating the plates for 2 h at 4°C with the indicated amounts of ^{125}I-labeled VLDL, either in the presence or absence of a 20-fold excess of unlabeled VLDL. Thereafter, the plates were washed two times with ice-cold PBS containing 0.1% (w/v) BSA, and subsequently washed with PBS without BSA. The ^{125}I-labeled VLDL bound to the HSPG-LPL complex was dissolved in 0.2 N NaOH for quantitation of the binding. High affinity binding was calculated by subtracting the amount of labeled VLDL that was

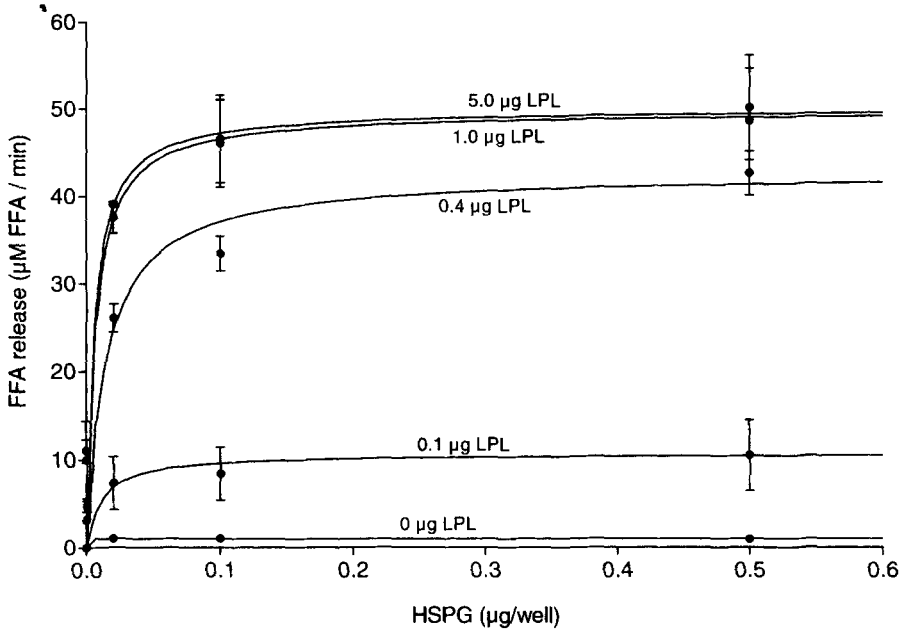

Fig. 2. Determination of optimal incubation conditions to study lipolysis of VLDL by HSPG-bound LPL. Plates were incubated with increasing concentrations HSPG and washed three times with PBS to remove unbound HSPG. Subsequently, the wells were incubated with 1% BSA in PBS to block nonspecific binding sites. Then, the plates were incubated with different amounts of LPL, as indicated. Plates were washed three times to remove unbound LPL and the lipolysis assay was started by adding control VLDL (TG 1.0 mM) to the preconditioned wells. After 10 min, the reaction was stopped by the addition of 1% Triton X-100. Free fatty acid release represents the mean ± SD for wells measured in triplicate.

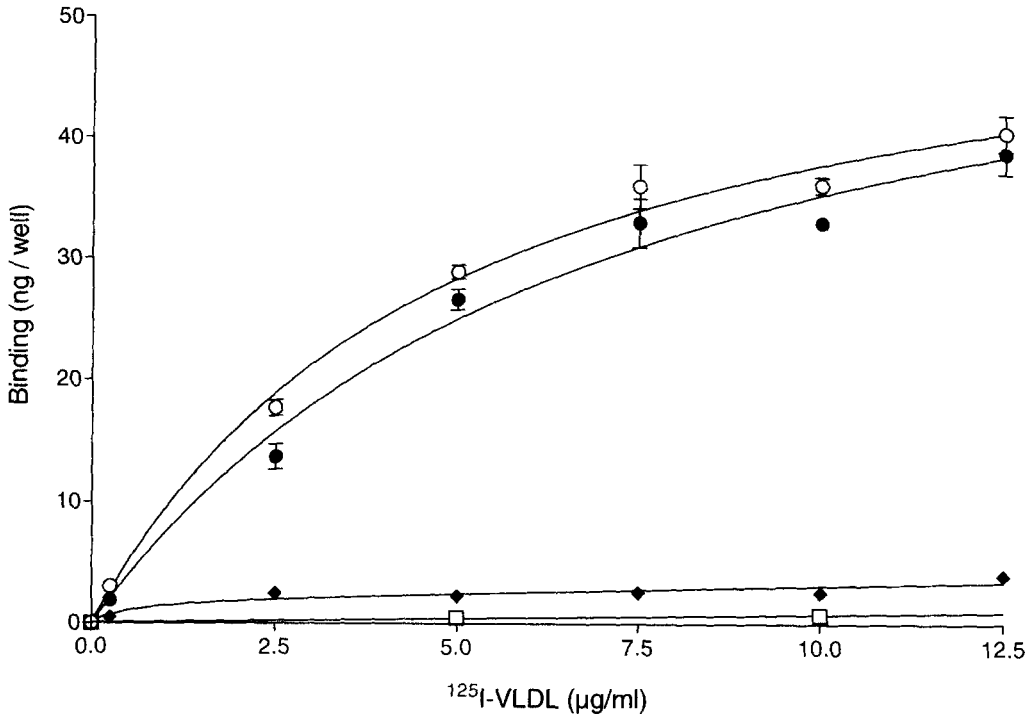

Fig. 3. Binding curve of ^{125}I-labeled VLDL to HSPG-bound LPL (●) and HSPG alone (□). After coating with 0.5 μg/well HSPG, nonspecific binding sites were blocked with PBS, 1% BSA, and treated with 1.5 μg LPL or 0 μg LPL per well, respectively. Plates were then incubated for 2 h at 4°C with different amounts of ^{125}I-labeled VLDL as indicated, either in the presence or absence of a 20-fold excess of unlabeled VLDL. High affinity binding to HSPG-LPL (●) was calculated by subtracting the amount of labeled VLDL that was specifically bound to the HSPG-LPL complexes (◆) from the amount of labeled VLDL that was bound in the absence of unlabeled VLDL (total binding) (○). VLDL binding to HSPG-bound LPL is expressed as ng of lipoprotein per well. Each value represents the mean ± SD of triplicate measurements.

bound to the HSPG-LPL complexes after incubation in the presence of a 20-fold excess of unlabeled VLDL (nonspecific binding) from the amount of labeled VLDL that was bound in the absence of unlabeled VLDL (total binding).

Binding of LPL. To measure the amount of LPL binding in the HSPG-LPL-coated wells, LPL was iodinated as described above. Wells were incubated with 0.5 μg HSPG for 18 h at 4°C, washed three times with PBS, and subsequently blocked for 1 h at 37°C with PBS containing 1% (w/v) essentially FFA-free BSA. Then, the wells were incubated with 1.5 μg ^{125}I-labeled LPL diluted in Tris-glycerol buffer for 1 h at 4°C. Thus, the pretreatment of the wells was performed exactly as described for the lipolysis assay with HSPG-bound LPL, with the exception that ^{125}I-labeled LPL was used. After washing the wells three times with ice-cold PBS, the ^{125}I-labeled LPL was dissolved in 0.2 N NaOH for quantitation. Of the initially added 1500 ng LPL, only 40 ng LPL proved to bind to the HSPG-coated wells (2.7%). Similar results were obtained when heparin was used to release LPL from the wells instead of 0.2 N NaOH.

RESULTS AND DISCUSSION

The present study was performed to develop an in vitro lipolysis assay using HSPG-bound LPL. The limited number of reports in literature describing a lipolysis assay with immobilized LPL used heparin-Sepharose columns as adhesive surface for LPL (5, 6). As this design has been shown to be technically difficult and poorly reproducible, we used plastic microtiter plates as adhesive surface for coating with HSPG and LPL.

The first objective was to determine the optimal incubation conditions for coating. Therefore, wells were incubated with increasing concentrations of HSPG (ranging from 0 to 3 μg/well) and LPL (ranging from 0 to 5 μg/well). As shown in **Fig. 2**, the FFA release increases with increasing amounts of HSPG, but at HSPG concentrations greater than 0.1 μg/well, lipolysis reaches a plateau indicating saturation. From Fig. 2 it is also obvious that lipolysis increases with increasing LPL concentrations in the second incubation step, showing saturation at an LPL concentration above 1.0 μg/well. All subsequent lipolysis experiments were carried out

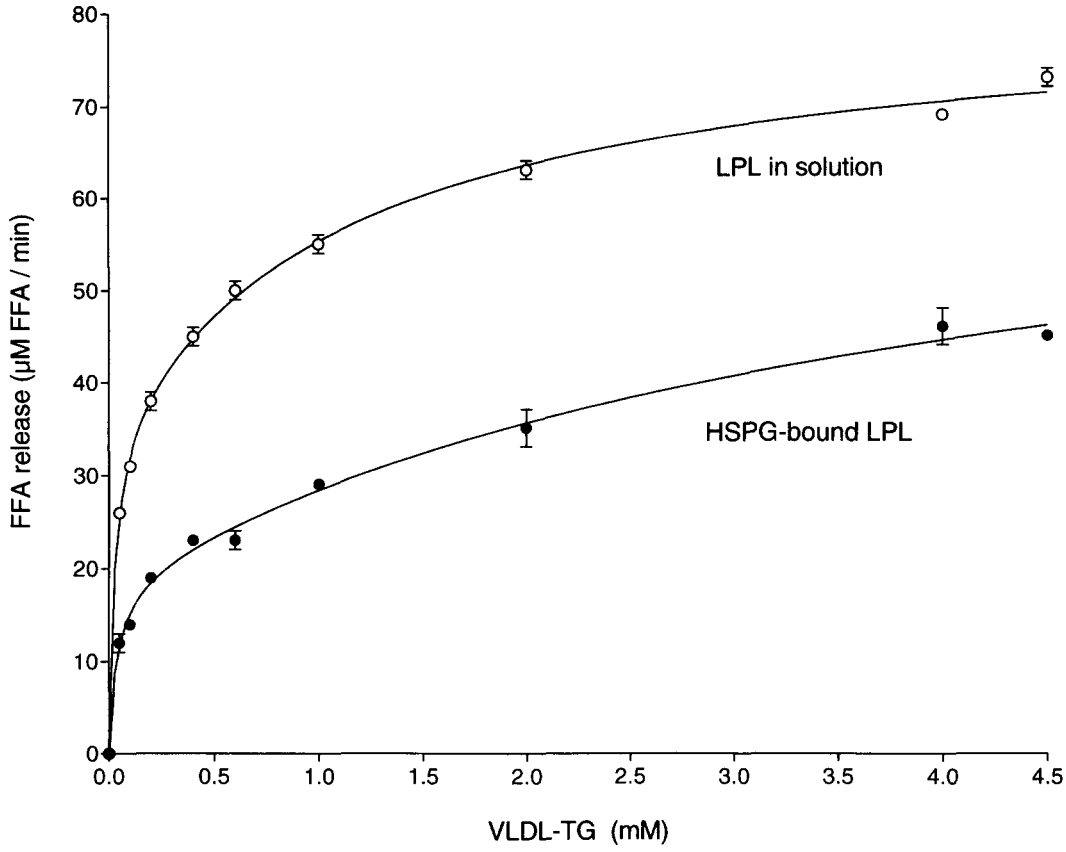

Fig. 4. Lipolysis experiments with HSPG-bound LPL (●) and LPL in solution (○) were carried out with increasing concentrations of VLDL-TG. With regard to the lipolysis assay with HSPG-bound LPL, wells were preconditioned with HSPG and LPL (Materials and Methods), washed, and subsequently incubated with various amounts of VLDL-TG in the presence of 1% BSA for 10 min at 37°C. The lipolysis assay with LPL in solution was performed by incubating VLDL-TG samples with LPL in solution for 6 min at 37°C. The reactions were stopped by addition of Triton X-100, vortexing, and cooling on ice. FFA concentrations were determined in triplicate.

in the saturated parts of the curves, using an HSPG concentration of 0.5 μg/well in the first incubation step and an LPL concentration of 1.5 μg/well in the second step.

To determine the VLDL binding characteristics under these conditions, preconditioned wells were incubated with increasing concentrations of ^{125}I-labeled VLDL. **Figure 3** clearly demonstrates that VLDL binds to the HSPG-LPL complexes of the preconditioned wells, whereas in the absence of LPL, VLDL binds very poorly to the HSPG-coated wells. This indicates that the current lipolysis assay represents lipolysis of VLDL after binding to immobilized LPL.

In order to assess kinetic parameters of the novel lipolysis assay and compare these with the conventional assay, lipolysis experiments with HSPG-bound LPL and LPL in solution were carried out with increasing concentrations of VLDL-TG. **Figure 4** shows the respective lipolysis curves. The apparent K_m values, as calculated by Lineweaver-Burk analysis, of the lipolysis assay with LPL in solution and HSPG-bound LPL are 0.20 ± 0.03 mM and 0.36 ± 0.11 mM VLDL-TG, respectively. The apparent V_{max} values of the lipolysis assay with LPL in solution and HSPG-bound LPL are 80 ± 3 μM FFA/min and 46 ± 4 μM FFA/min, respectively. Taking into account the differences in LPL availability, corresponding apparent V_{max} values of the lipolysis assay with LPL in solution and HSPG-bound LPL are 11.4 ± 0.1 μM FFA/min·ng LPL and 1.2 ± 0.1 μM FFA/min·ng LPL, respectively. Thus, the conventional assay with LPL in solution yields a lower K_m and higher V_{max} value as compared to the novel assay with HSPG-bound LPL, which is in agreement with previous studies (5). As lipolysis kinetic studies are performed preferably with substrate concentrations in the K_m range, subsequent lipolysis experiments were performed in the VLDL-TG range of 0.2–0.6 mM.

To determine whether, under these assay conditions, HSPG-LPL-mediated lipolysis of VLDL occurs by LPL molecules that bound specifically to HSPG, precondi-

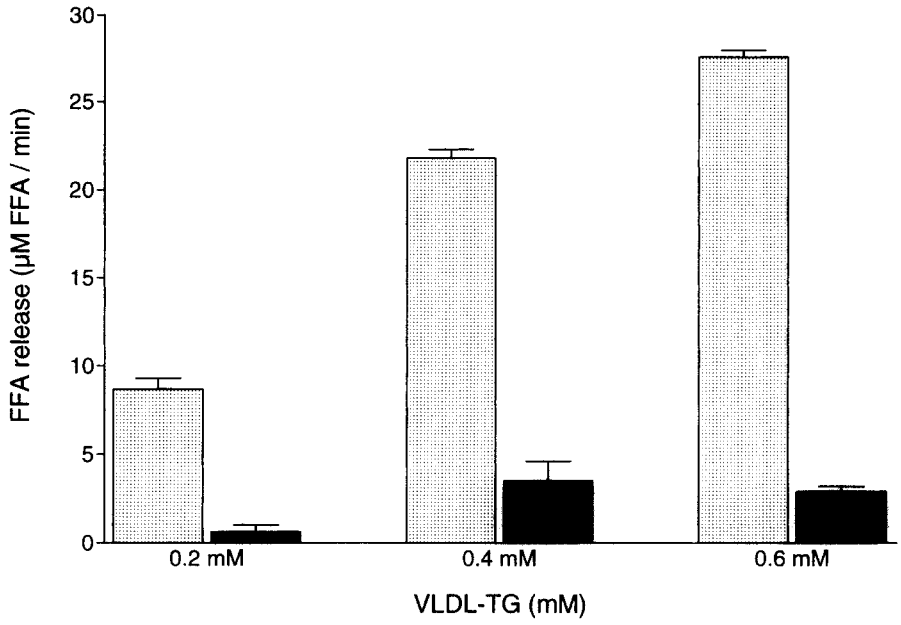

Fig. 5. Effect of heparinase treatment on HSPG-LPL mediated lipolysis. HSPG-LPL-coated plates were incubated with PBS containing heparinase I/III (■), or PBS without heparinase (■) at 37°C for 20 min. Then, the wells were washed three times. Lipolysis was carried out by incubating VLDL at three different VLDL-TG concentrations for 10 min at 37°C.

tioned wells were incubated with heparinase in order to hydrolyze heparan sulfates (3, 12). As shown in **Fig. 5**, treatment of HSPG-LPL-coated wells with heparinase reduced lipolysis to approximately 10% of the normal lipolysis rate. These results indicate that at least 90% of the LPL activity is indeed derived from LPL bound to heparan sulfate chains.

It is known that LPL can detach from endothelium during lipolysis (24, 25). To check whether LPL would detach from the HSPG-complexes in the presence of VLDL, the following control experiment was performed. Wells were preconditioned with HSPG and ^{125}I-labeled LPL and subsequently incubated in the presence or absence of VLDL (see Materials and Methods). After washing the wells, the remaining amount of ^{125}I-labeled LPL was released from the HSPG-complexes for quantitation. No differences were noted between the wells incubated with VLDL-TG and the wells incubated with the lipoprotein-free buffer alone (data not shown). Thus, incubation of HSPG-LPL-coated wells with VLDL does not result in detachment of LPL from the HSPG-complexes under the conditions applied.

To test the reproducibility of the lipolysis assay, repetitive lipolysis experiments were performed with VLDL isolated from a healthy normolipidemic subject. The first lipolysis experiment was carried out with freshly isolated VLDL; the second and third experiments were carried out with VLDL isolated from the same serum but stored for 1 and 4 weeks at −80°C (see Materials and Methods). Nearly identical rates of FFA release were observed in the three consecutive experiments (**Table 1**). The inter-assay coefficients of variance ranged from 3 to 11%, depending on the substrate concentration used. Thus, the lipolysis assay with HSPG-bound LPL appears to be reproducible and storage at −80°C under well-defined conditions does not affect lipolysis rates. Inter-individual variation in lipolysis was assessed by performing lipolysis experiments with separate VLDL samples, isolated from four normolipidemic apoE3 homozygous subjects. The mean inter-individual coefficient of variance was calculated to be 18% (Table

TABLE 1. Reproducibility and biological variation of VLDL-TG lipolysis using HSPG-bound LPL

Concentration of VLDL-TG Used in Assay	Storage at −80°C (weeks)				Control Subjects (n = 4)				
	0	1	4	CV_{ia}[a]	A	B	C	D	CV_{ii}[b]
mmol/l	*mmol FFA/l*			%	*mmol FFA/l*				%
0.20	0.19	0.16	0.16	11	0.22	0.15	0.17	0.22	18
0.40	0.33	0.27	0.29	10	0.35	0.24	0.24	0.34	21
0.60	0.42	0.40	0.41	3	0.44	0.35	0.31	0.42	15

Lipolysis rates are expressed as mmol FFA/l after 10 min incubation in HSPG–LPL-coated wells.

[a] CV_{ia}, inter-assay coefficient of variance.

[b] CV_{ii}, inter-individual coefficient of variance.

1). The intra-assay coefficient of variance was 5%. Although the variation between different lipolysis experiments (inter-assay coefficient of variance) is acceptable, it is recommended to use an internal standard in each series of lipolysis experiments, e.g., a plasma pool stored at −80°C after cryopreservation from which VLDL can be isolated for each new series of lipolysis experiments.

The potential benefits of this novel lipolysis assay over the conventional assay with LPL in solution remain to be established. However, preliminary results indicated that VLDL isolated from patients with different apoE mutations showed different lipolysis efficiencies by HSPG-bounds LPL which were paralleled by differences in binding of VLDL to the HSPG-LPL complex (26). In contrast, the conventional lipolysis assay with LPL in solution did not detect differences in lipolysis. Thus, the current assay with HSPG-bound LPL may provide the proper experimental tool to detect differences in lipolysis efficiency if an altered interaction between VLDL and HSPG-LPL complex is expected. This may be the case in endogenous hypertriglyceridemia. In vitro lipolysis experiments with hypertriglyceridemic VLDL using LPL in solution did not demonstrate an impaired lipolysis as compared to VLDL isolated from healthy subjects (27). However, because apoE and apoC may modulate the binding of triglyceride-rich lipoproteins to heparan sulfate (15), it is speculated that in patients with endogenous hypertriglyceridemia, whose VLDL contain an increased apoC content per particle, a reduced binding to HSPG-bound LPL and therefore a reduced lipolysis efficiency may be expected. The current assay with HSPG-bound LPL may provide the proper experimental tool to address this issue in the future.

The current lipolysis assay with HSPG-bound LPL presents a simple method to study lipolysis of VLDL. An important improvement is the preserved interaction among VLDL, LPL, and HSPG. Although this system shows a better resemblance to the in vivo situation than the conventional assay with free LPL, there are still some important differences. FFAs and other lipolysis products cannot be disposed into the underlying tissue but remain in the proximity of the enzyme–substrate complex. In addition, LPL is bound to HSPG alone whereas in the normal situation, LPL is bound to a different proteoglycans and non-proteoglycan LPL-binding proteins (28, 29). A lipolysis assay using LPL bound to endothelial cells, as has been described by Saxena, Witte, and Goldberg (30), therefore has theoretical advantages over the present cell-free system with HSPG-bound LPL. However, several practical problems have to be solved before this concept of endothelial-bound LPL can be used for measuring the kinetics of VLDL-TG lipolysis. In particular, the exact number of cells in the system over a longer period of time (months to years) and the time- and cell phase-dependent expression of extracellular matrix proteins may affect the reproducibility of the assay (31).

We conclude that the current lipolysis assay presents a simple and reproducible method to study lipolysis of VLDL, whereby the interaction among VLDL, LDL and HSPG is preserved.

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