Heparin-binding defective lipoprotein lipase is unstable and causes abnormalities in lipid delivery to tissues

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Lipoprotein lipase (LpL) binding to heparan sulfate proteoglycans (HSPGs) is hypothesized to stabilize the enzyme, localize LpL in specific capillary beds, and route lipoprotein lipids to the underlying tissues. To test these hypotheses in vivo, we created mice expressing a human LpL minigene (hLpL^{HBM}) carrying a mutated heparin-binding site. Three basic amino acids in the carboxyl terminal region of LpL were mutated, yielding an active enzyme with reduced heparin binding. Mice expressing hLpL^{HBM} accumulated inactive human LpL (hLpL) protein in preheparin blood. hLpL^{HBM} rapidly lost activity during a 37°C incubation, confirming a requirement for heparin binding to stabilize LpL. Nevertheless, expression of hLpL^{HBM} prevented the neonatal demise of LpL knockout mice. On the LpL-deficient background hLpL^{HBM} expression led to defective targeting of lipids to tissues. Compared with mice expressing native hLpL in the muscle, hLpL^{HBM} transgenic mice had increased postprandial FFAs, decreased lipid uptake in muscle tissue, and increased lipid uptake in kidneys. Thus, heparin association is required for LpL stability and normal physiologic functions. These experiments confirm in vivo that association with HSPGs can provide a means to maintain proteins in their stable conformations and to anchor them at sites where their activity is required.

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Introduction

Lipoprotein lipase (LpL) hydrolyzes circulating triglyceride (TG) into FFAs while it is bound to the luminal surface of endothelial cells (1). Several lines of evidence suggest that this interaction is highly dependent on LpL binding to endothelial cell surface heparan sulfate proteoglycans (HSPGs): (a) LpL is a high affinity heparin-binding protein (2); (b) LpL is released into the bloodstream of humans and animals by intravenous injection of heparin; (c) heparinase treatment of endothelial cells markedly reduces LpL binding to endothelial cells (3); and (d) high affinity LpL-binding proteoglycans (4) and heparan-sulfate oligosaccharides (5, 6) have been isolated from cultured endothelial cells.

Heparin binding is important for biological actions of a number of proteins including enzymes, growth factors, cytokines, and coagulation proteins. In some situations, association with HSPGs appears to concentrate proteins near signaling and endocytosis receptors (7). This, for example, allows maximum function of bFGF (8). Heparin binding had been postulated to require consensus sequences containing clusters of basic amino acids containing the sequences XBBXBX and XBBBXXBX (9). However, more recent reviews of heparin-binding proteins have concluded that this simplistic approach to defining heparin-binding regions without consideration of the tertiary and quaternary structure of proteins has often been incorrect (10, 11).

Aside from its importance in lipid/lipoprotein metabolism, LpL is a model protein for studying physiologic implications and biochemistry of heparin binding and protein dimerization. LpL association with HSPGs is postulated, from in vitro data, to affect LpL metabolism at three separate steps. LpL binding to HSPGs on the luminal side of endothelial cells concentrates LpL in sites of enzyme synthesis like the adipose and muscles. This is hypothesized to promote local uptake of LpL-generated fatty acids into those tissues. A second role of LpL binding to HSPGs may be to stabilize the enzyme on its endothelial-binding site. In vitro data have demonstrated that LpL is rapidly inactivated at 37°C, and this loss of activity is partially prevented by inclusion of heparin in the incubation. Finally, heparin binding may be required for efficient removal of LpL from the bloodstream. Support for this role of heparin binding comes from experiments showing that heparin injection into animals delays clearance of LpL by the liver (12).

LpL has several clusters of basic amino acids that were thought to represent heparin-binding domains. When a postulated heparin-binding region in the amino terminal half of LpL was mutated, LpL-heparin interaction

was decreased but not eliminated (13–15). This suggested that a second site was involved and probably represented the major heparin-binding region. By creating chimeric molecules using regions of LpL and hepatic lipase, it was shown that high affinity heparin binding requires the carboxyl terminal region of LpL (16, 17); this region does not contain a consensus heparin-binding sequence. While some in vitro experiments suggested that the carboxyl-terminal region of LpL contains a major heparin-binding region (18), other experiments using bacterially expressed LpL fragments failed to confirm this (19). Thus, although heparin binding is a characteristic of LpL, the region(s) of the molecule required for HSPG binding has not been defined in vivo and the in vitro experiments are conflicting.

To determine whether the carboxyl terminal region of human LpL (hLpL) is important for HSPG binding in vivo and to assess the biological effects of having heparin-binding mutated LpL, we produced mutations in the carboxyl terminal region of hLpL corresponding to mutations in avian LpL cDNA that lead to a significant loss in heparin binding (18) and studied their physiologic consequences in a transgenic mouse model.

Methods

Construction of human heparin-binding mutated LpL (hLpL-HBM). A PCR-based site-specific mutagenesis of the muscle creatine kinase–hLpL (MCK-hLpL) minigene (20) was performed using a kit (Stratagene, La Jolla, California, USA) and the following primers: Primer 1: GCT TCG CCA TTC AGA ACA TCA ATG TAA ATG CAG GAG AGA CTC AG, primer 2: CTG AGT CTC TCC TGC ATT TAC ATT GAT GTT CTG AAT GGC GAA GC. This procedure converted the coding regions for amino acids 403, 405, and 407 of the hLpL cDNA from Arg[403] to Asn[403], Arg[405] to Asn[405], and Lys[407] to Asn[407].

The mutated hLpL[HBM] cDNA was cloned in a pcDNA3.1 vector in which expression is driven by the cytomegalovirus promoter. This plasmid was stably transfected in Chinese hamster ovary cells (CHO-K1) as described (18). The nonmutated hLpL expressing CHO cells were a generous gift by N. Yamada (Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan) (21). Cells were maintained as described previously (18). For LpL activity assays confluent cells were incubated with serum-free medium (DMEM 2% BSA) for 2 hours. The media were collected and immediately frozen at –70°C.

Generation of hLpL[HBM] transgenic mice. The purified MCK-hLpL[HBM] minigene was microinjected into the pronuclei of fertilized mouse eggs taken from superovulated (C57BL/6J × CBA/J) F1 females as described (22). Founder animals were crossed with C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine, USA). hLpL[HBM] transgenic mice were then bred with heterozygote LpL knockout mice that had been bred more than five generations onto C57BL/6 (LpL1, ref. 23). Pups, which carried the hLpL[HBM] transgene as well as the disrupted mouse LpL allele, were crossed again with

LpL1 mice. This cross resulted in the generation of 12.5% of pups that were homozygous for the LpL knockout allele and heterozygous for the hLpL[HBM] transgene. hLpL transgenic mice were bred in the same manner. From this breeding scheme, each line of mice had ≥ 93.75% C57BL/6 background.

Genotyping of transgenic mice. Tail-tip DNA was screened by PCR. The genotype at the mouse LpL locus was analyzed by the 3 primer PCR as described. The hLpL[HBM] and the hLpL transgenes were detected by utilizing the hLpL PCR (24).

LpL mass and activity measurements. To obtain postheparin plasma, fasted mice were bled 5 minutes after a tail vein injection of 100 U heparin/kg body weight (Elkins-Sinns Inc., Cherry Hill, New Jersey, USA). Human and murine LpL protein was measured by ELISA as described previously by Peterson et al. (25). LpL activity was measured by the method described by Hocquette et al. (26). To distinguish lipolysis mediated by hLpL, mouse LpL, and hepatic lipase, the mouse plasma samples were assayed in the presence of an mAb against hLpL (27) and under high salt conditions (1M NaCl final concentration).

Heparin-Sepharose chromatography. Affinity chromatography was performed using an FPLC-System (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) with a 1 ml Hi-Trap heparin-Sepharose column (Amersham Pharmacia Biotech) at 4°C essentially as described (18). Murine and hLpL concentrations in the fractions were determined by ELISA as described.

Gel filtration of postheparin plasma. Five hundred microliters pooled postheparin plasma from male mice (n = 4 of each genotype, hLpL[HBM]/LpL0 and hLpL/LpL0) was chromatographed by FPLC using two Superose 6 columns in series (Amersham Pharmacia Biotech) as described (28). Twenty-five fractions of 1.08 ml were collected and assayed for LpL activity, hLpL mass, and cholesterol as described above.

Plasma lipid and lipoprotein analysis. The mice were fed a chow diet (4.5% fat, wt/wt). Unless stated differently all plasma samples were collected after 8 hours of daytime fasting. Plasma TG and cholesterol were determined with kits (Sigma-Aldrich, St. Louis, Missouri, USA) in duplicates. For lipoprotein analysis individual mouse plasma samples (60 μl) were centrifuged twice in a Beckman TLA 100 rotor (Beckman Instruments Inc., Palo Alto, California, USA) as described (29). FFAs were determined after overnight fasting and in the postprandial state after gavage of 100 μl corn oil (1 hour, 2 hours, and 3 hours postprandial). FFAs were measured in duplicates utilizing a commercial kit (NEFA C; Wako Pure Chemicals Industries Ltd., Osaka, Japan).

Northern blot. RNA was prepared from 8-week-old hLpL[HBM]/LpL0 and hLpL/LpL0 female mice using a kit (TRIzol Reagent; Life Technologies Inc., Rockville, Maryland, USA). The blot was hybridized with a probe spanning the ~500 bp RsRII/HindIII fragment from the hLpL minigene and then autoradiographed. A second hybridization with an oligonucleotide complementary to 18S rRNA (30) was performed.

LpL stability. Serum-free cell culture media from CHO cells expressing either nonmutated hLpL or hLpL[HBM] were harvested as described before. To one batch of media, 5 U/ml heparin (Elkins-Sinns) was added. Eighty microliters of the media was assayed for activity just before incubation at 37°C in a water bath and then after 15 minutes, 30 minutes, and 60 minutes as described above. In another experiment, two sets of three wells each on a 6-well plate of hLpL and hLpL[HBM] expressing CHO cells were used. The first set of both cell types was incubated for 4 hours with DMEM 2% BSA. The media were collected and frozen at –70°C. Thereafter, the cells were washed twice with ice cold PBS and then incubated with DMEM 2% BSA + 20 U/ml heparin for 30 minutes at 4°C. The media were collected and frozen. The second set of cells was only incubated with the heparin containing media as above. LpL activity was assessed in triplicates using 80 µl aliquots of media. LpL stability was also assessed in postheparin plasma from hLpL/LpL0 and hLpL[HBM]/LpL0 mice. Five microliters of 1:5 in water-diluted postheparin plasma was assayed for activity just before incubation at 37°C and after 30 minutes, 60 minutes, and 90 minutes.

LpL in skeletal muscle and kidneys. The mice were perfused with 20 ml PBS prior to removal of tissues. Quadriceps, muscles, and kidneys (100 mg wet weight) were obtained from 4- to 6-month-old mice expressing only hLpL[HMB] or hLpL. The muscles were then minced and incubated at 4°C with DMEM 2% BSA on a rocker. After 2 hours the media were collected and the tissues were washed with PBS and then homogenized as described by Hocquette et al. (26). The kidney tissues were directly homogenized (26) after removal. LpL mass in the media and tissues was measured as above.

Turnover of rat chylomicrons, Intralipid, and palmitate. Rat chylomicrons were collected and purified from the mesenteric lymph of male Wistar rats exactly as described (31) with the exception that the animals were fed 500 µCi of (^{3}H)triolein (specific activity 21 Ci/mmol, Amersham Pharmacia Biotech) and 10 µCi of (^{14}C)cholesterol (specific activity 1 mCi/ml, Amersham Pharmacia Biotech) dissolved in 500 µl corn oil to obtain TG and cholesterol-labeled chylomicrons. Intralipid (Kabi Pharmacia Inc., Clayton, North Carolina, USA) emulsion was labeled with 40 µCi (^{3}H)cholesteryl oleyl ether and 10 µCi (^{14}C)triolein (Amersham Pharmacia Biotech) exactly as described

by van Bennekum et al. (32). One hundred seventy microliters of the chylomicron emulsion containing 1.1×10^6 ^{3}H decays per minute (dpm) and 2×10^4 ^{14}C dpm were injected into hLpL[HBM]/LpL0 (*n* = 5) and hLpL/LpL0 (*n* = 5) male mice at the age of 10–12 weeks via tail vein. The Intralipid emulsion was diluted in sterile saline prior to intravenous injection to 10^6 dpm (^{3}H) cholesteryl oleyl ether and 3×10^5 dpm (^{14}C)triolein in a volume of 100 µl. Intralipid metabolism was studied in four hLpL[HBM]/LpL0 and four hLpL/LpL0 female mice at the age of 14–16 weeks. Eighty microliters of blood was withdrawn from retroorbital plexus 2 and 4 minutes after injection. Ten minutes after injection mice were bled by cardiac puncture and then perfused with 10 ml PBS. One hundred microliters of the plasma from the 10-minute time point of the chylomicron turnover study was lipid extracted and TLC was performed using standard techniques. After perfusion the tissues were removed, homogenized, total lipids were extracted, and were counted as described (33). Fatty acid turnover utilized (^{3}H)palmitate in ethanol (NEN Life Science Inc., Boston, Massachusetts, USA; specific activity 36.3 Ci/mmol) complexed to fatty acid free BSA (Sigma-Aldrich) as described (34). 2.2×10^6 dpm palmitate were injected via tail vein into four hLpL[HBM]/LpL0 and four hLpL/LpL0 male 5- to 6-month-old mice. The mice were bled from retro-orbital plexus at 15 seconds, 1 minute, 2 minutes, and 3 minutes after injection, and the plasma decay was determined.

Histological analysis. Quadriceps, soleus, and psoas muscles were dissected from 8-month-old female hLpL[HBM]/LpL2 and wild-type littermates and fixed in formaldehyde. Histological analyses including electron microscopy were performed as described (33).

Results

Generation of transgenic mice. Transgenic mice were generated that express the hLpL[HBM] minigene in the muscle. The expression of hLpL[HBM] was confirmed by measuring hLpL activity and mass in plasma. As shown in Figure 1a, hLpL protein was found in postheparin plasma from these mice. No hLpL protein was detected in the plasma of wild-type littermates. Surprisingly, large amounts of hLpL[HBM] were also found in preheparin plasma. In more than 20 hLpL[HBM]-expressing mice, plasma preheparin LpL was greater than 1.2 µg/ml. Thus, hLpL[HBM] differed

Figure 1
hLpL mass and activity of hLpL[HBM] transgenic mice. (**a**) hLpL mass in pre- and postheparin plasma of hLpL[HBM] and hLpL transgenic mice. Preheparin plasma was collected immediately before intravenous injection of 100 U heparin/kg. Five minutes later postheparin plasma was obtained. hLpL mass was determined by ELISA as described in Methods. (**b**) hLpL[HBM] and murine LpL (mLpL) activity in postheparin plasma of hLpL[HBM] transgenic mice and their wild-type littermates. A monoclonal antibody against hLpL (28) was utilized to distinguish between hLpL[HBM] and mLpL activity.
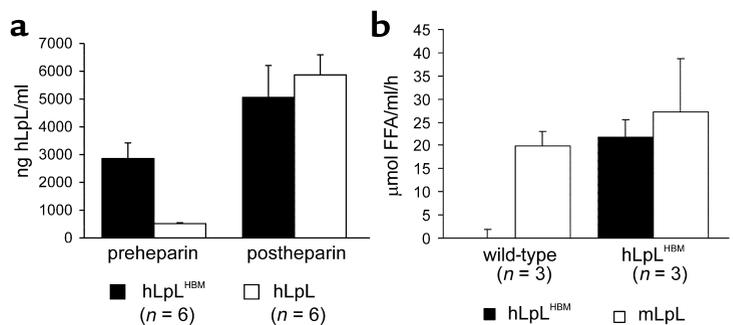
Figure 2

Heparin-affinity chromatography and gel filtration of mouse plasma. (**a**) Preheparin plasma from hLpL[HBM] transgenic mice was collected and applied to a heparin-affinity gel. LpL was eluted with a NaCl gradient and hLpL was measured by ELISA. (**b**) Postheparin plasma from hLpL[HBM] transgenic mice was chromatographed on a heparin-affinity gel. Human and murine LpL masses were determined by ELISA. The amounts of LpL are given as a percentage of eluted LpL per fraction. (**c**) Pooled postheparin plasma from hLpL[HBM]/LpL0 mice was gel filtered on an FPLC system with two Superose 6 columns in series. LpL activity and cholesterol were determined in each fraction. (**d**) Pooled postheparin plasma from hLpL/LpL0 mice was gel filtered and analyzed as described in **c**.
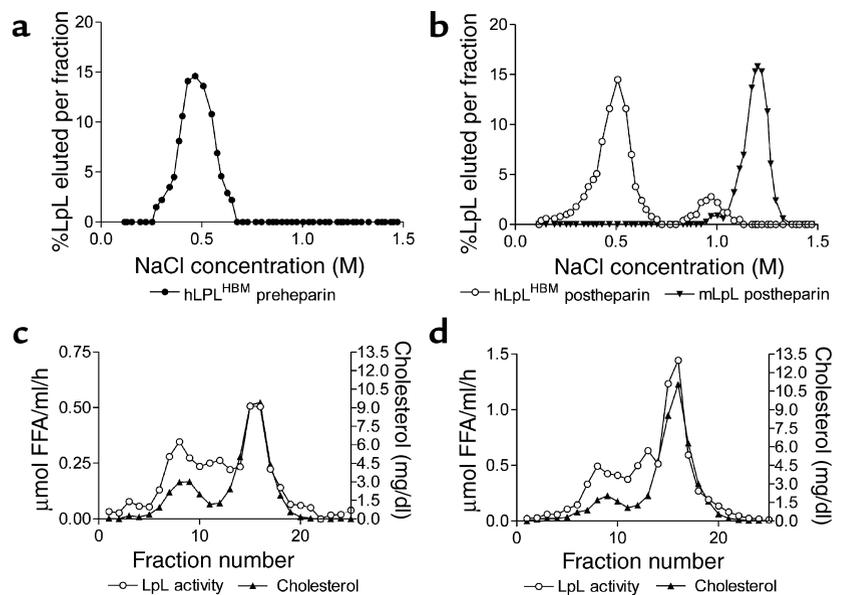
in its metabolism such that the protein was defective in its ability to remain bound to the endothelium or in its clearance from the bloodstream.

hLpL activity in postheparin plasma of hLpL[HBM] transgenic mice was 21.7 ± 3.8 μmol FFA/ml/h, an amount that was similar to endogenous mouse LpL in these animals. Wild-type mouse postheparin plasma contained less than 0.1 ± 1.9 μmol FFA/ml/h of hLpL activity (Figure 1b). Despite the large amount of protein, no hLpL activity was detected in the preheparin plasma of hLpL[HBM]-expressing mice.

Heparin-affinity chromatography of pre- and postheparin plasma from hLpL[HBM] transgenic mice. The preheparin plasma hLpL[HBM] protein eluted at ~0.4 M NaCl, Figure 2a. A second peak of hLpL[HBM] protein eluted at ~0.9 M NaCl when postheparin plasma was chromatographed (Figure 2b). Murine LpL in the postheparin plasma eluted at ~1.2 M NaCl. At ~0.9 M there was a small region where the hLpL[HBM] elution overlapped a shoulder of the murine LpL elution; this may represent heterodimers of mouse LpL and hLpL[HBM]. These data demonstrate that plasma from mice expressing hLpL[HBM] contains a low affinity inactive protein in preheparin blood. Postheparin plasma appears to contain hLpL monomers and dimers, both of which are defective in heparin binding.

Breeding of hLpL[HBM] onto the LpL0 background. We next tested whether the hLpL[HBM] transgene would prevent the neonatal demise of LpL0 mice; these mice die within 24 hours of birth. All pups with the hLpL[HBM]/LpL0 genotype survived and appeared normal. Postheparin plasma from hLpL[HBM]/LpL0 mice contained 16.1 ± 3.1 μmol FFA/ml/h of hLpL activity. This is comparable to the hLpL activity found in mice expressing nonmutated hLpL using the MCK promoter (ref. 20, 23.8 μmol FFA/ml/h).

Interaction of hLpL[HBM] and hLpL with lipoproteins in postheparin plasma of mice on the LpL0 background. As shown in

Figure 2c (hLpL[HBM]/LpL0) and Figure 2d (hLpL/LpL0), LpL activity coeluted with cholesterol-rich lipoproteins in both genotypes. In contrast to LpL in human postheparin plasma (35), the majority of LpL activity was associated with HDL, presumably because of the lack of sufficient amounts of apoB-containing lipoproteins to bind all the LpL. hLpL mass in both genotypes also coeluted with LDL and HDL; in the hLpL[HBM] there was no significant non-lipoprotein-associated LpL protein (data not shown). Thus, the carboxyl-terminal mutation in hLpL[HBM] did not significantly alter the enzyme's ability to bind to lipoproteins.

Northern blot. Northern blot analysis was performed to compare expression levels of the transgenes in hLpL[HBM] and hLpL mice. In skeletal muscle (Figure 3, lanes 1 and 2, 90-minute exposure) and heart (lanes 3 and 4, 16-hour exposure), more LpL mRNA was found in tissues from the hLpL[HBM] expressing mice (lanes 2 and 4). Neither transgene was expressed in kidneys (lanes 5 and 6, 16-hour exposure) or lungs (lanes 7 and 8).

Plasma lipids. On the wild-type background hLpL[HBM] did not significantly alter plasma TG or cholesterol levels (TG levels were 50 ± 5 in hLpL[HBM]/LpL2 females and 51 ± 5 in LpL2 female littermates, *n* = 4 each; TG was 65 ± 17 in hLpL[HBM]/LpL2 males, *n* = 4, and 81 ± 17 in LpL2 male littermates, *n* = 3). However, on the heterozygous LpL knockout background hLpL[HBM] significantly reduced plasma TG levels (Table 1). In addition, plasma lipid values obtained from LpL1 littermates without the transgenes are shown; note that the littermates derived from breeding of hLpL and hLpL[HBM] had no significant differences in lipids. This suggests that there were not underlying genetic differences in the two lines of mice. On the homozygous LpL knockout background hLpL[HBM] mice were compared with hLpL transgenic mice and wild-type littermates. Both male and female hLpL[HBM]/LpL0 mice had higher levels of plasma
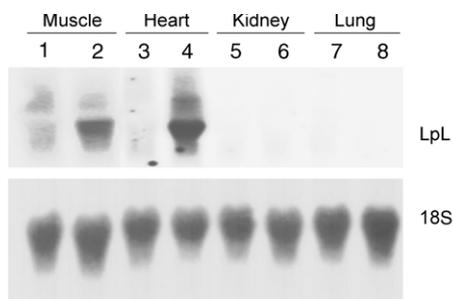
Figure 3

LpL RNA tissue levels of hLpL[HBM] and hLpL transgenic mice on the LpL knockout background. Total RNA of the indicated tissues of hLpL[HBM]/LpL0 and hLpL/LpL0 mice was separated on a 1% agarose gel, blotted to a nylon membrane, and hybridized with the ~500 bp RsrII/HindIII fragment of the hLpL minigene. Lanes 1 and 2 show a 90-minute autoradiography of the blot while the lanes 3–8 show a 16-hour exposure. hLpL[HBM] RNA is in lanes 2, 4, 6, and 8; hLpL-RNA is in lanes 1, 3, 5, and 7. For control, 18S RNA was hybridized with a complementary oligonucleotide.

TG than either hLpL/LpL0 or wild-type mice. This was due to an approximately twofold increase in VLDL TG ($P < 0.001$ compared with hLpL/LpL0). Plasma cholesterol was similar in the two types of mice. In both female and male hLpL[HBM]/LpL0 mice VLDL cholesterol was significantly higher than in hLpL/LpL0 ($P < 0.03$).

LpL stability. The observation that there was a large amount of inactive LpL protein in preheparin plasma of mice expressing the hLpL[HBM] transgene suggested that this enzyme was less stable than hLpL. To test this, the stability of hLpL[HBM] activity was analyzed in three ways. Figure 4a shows that medium from hLpL[HBM]-expressing CHO cells lost lipolytic activity quicker than that from hLpL-expressing cells. Heparin stabilized both

hLpL and hLpL[HBM], but the mutated LpL still lost activity more quickly (Figure 4b). When residual LpL activity produced by transfected cells was assayed after a 4-hour incubation at 4°C, more than 80% of hLpL remained while less than 15% of the hLpL[HBM] activity was recovered (Figure 4c). Finally, we compared the LpL-stability in postheparin plasma of mice expressing either nonmutated hLpL or hLpL[HBM] on the LpL0 background (Figure 4d). After 30 minutes of incubation at 37°C, hLpL[HBM] postheparin plasma contained an undetectable amount of activity, whereas postheparin plasma containing hLpL retained more than 70% of the initial LpL activity. Therefore in addition to decreasing LpL binding to heparin, mutation of the three basic amino acids in the carboxyl-terminus of LpL leads to a marked decrease of the enzyme's stability.

Release of hLpL and hLpL[HBM] from muscle. To determine if the mutation in heparin binding also affects LpL association with muscle, the amount of mutated and hLpL dissociated from the muscle in 4°C media was assessed. Total immunoreactive LpL protein was greater in the hLpL[HBM] expressing muscle (2.3 ± 1.0 versus 1.0 ± 0.3 µg/100 mg of tissue, $P < 0.05$, $n = 4$ for each group). More hLpL[HBM] protein dissociated into the medium during the incubation (0.9 versus 0.3 µg/100 mg of muscle), and this was a greater percent of total LpL protein than was found in the hLpL-expressing tissues. Thus, hLpL[HBM] readily dissociated from binding sites within the muscle, and this was responsible for much of the hLpL[HBM] protein in preheparin blood.

Effects of hLpL[HBM] expression on postprandial lipemia. The role of heparin binding in the physiological targeting of lipoprotein lipids was tested in these mice. Female mice had similar plasma levels of FFAs after overnight fasting (1.35 ± 0.3 mmol FFA/l in hLpL[HBM]/LpL0,

Table 1
Plasma lipids on the LpL knockout background

	Background		sex	n	hLpL[HBM]	n	littermates	n	hLpL	n	littermates
TGs											
	LpL1	Plasma	f	6	57.5 ± 8.8[A]	6	106 ± 18	9	60.2 ± 14.7[A]	5	105.8 ± 9.7
			m	8	87.6 ± 15.2[A,B]	4	128.2 ± 12.8	8	53.6 ± 5.3[A]	6	125.3 ± 29.3
	LpL0	Plasma	f	18	91.4 ± 19.5[C]			10	54.3 ± 9.8		
			m	15	102.9 ± 14.7[C]			14	61.5 ± 18.6		
		VLDL	f	18	55.3 ± 11.4[C]			10	24.6 ± 5.8		
			m	15	73.9 ± 14.2[C]			14	34.4 ± 14.9		
		LDL	f	18	7.8 ± 2.2			10	8.0 ± 1.3		
			m	15	10.3 ± 3.9			14	10.5 ± 6.4		
		HDL	f	18	1.6 ± 1.5			10	3.0 ± 2.3		
			m	15	3.1 ± 6.5			14	3.3 ± 7.2		
Cholesterol											
	LpL1	Plasma	f	6	74.7 ± 10.4	6	65.9 ± 15.7	9	71.4 ± 11.0	5	83.9 ± 19.3
			m	8	78.3 ± 13.4	4	83.5 ± 8.1	8	68.7 ± 14.5	6	83.7 ± 20.9
	LpL0	Plasma	f	18	71.5 ± 11.9			10	68.1 ± 19.2		
			m	15	77.2 ± 19.7			14	75.5 ± 15.8		
		VLDL	f	18	12.3 ± 1.6[D]			10	10.5 ± 2.0		
			m	15	9.7 ± 5.2[D]			14	5.0 ± 3.1		
		LDL	f	18	19.1 ± 5.3			10	17.2 ± 3.9		
			m	15	11.3 ± 2.6			14	12.0 ± 3.7		
		HDL	f	18	43.2 ± 7.9			10	42.0 ± 15.3		
			m	15	45.2 ± 11.7			14	48.1 ± 10.6		

Littermates, mice not carrying the transgene [A]$P < 0.002$ vs. littermates; [B]$P < 0.001$ vs. hLpL/LpL1; [C]$P < 0.001$ vs. hLpL/LpL0; and [D]$P < 0.03$ vs. hLpL/LpL0.

LpL stability. (**a**) Media from CHO cells expressing nonmutated hLpL (filled squares) and hLpL[HBM] (open circles) were assayed for LpL activity before and at the indicated time points during incubation at 37°C. The activity before the incubation is set as 100%. (**b**) 5 U heparin/ml were added to the cell culture media, then the experiment was performed as in **a**. (**c**) The stability of hLpL[HBM] (open bars) associated with the cell surface of CHO cells was compared with hLpL (filled bars). The initial cell-associated LpL activity was determined by incubating the cells with 20 U/ml of heparin for 30 minutes at 4°C. This was set as 100%. Then LpL activity in heparin-free media after a 4-hour incubation at 4°C with the cells is shown. The third bar indicates the activity released from the cell surface with heparin after the 4-hour incubation described before. (**d**) Stability of postheparin plasma from hLpL[HBM]/LpL0 was compared with hLpL/LpL0. The samples were assayed for LpL activity before incubation at 37°C and at the indicated time points. The initial activity was set to 100%.
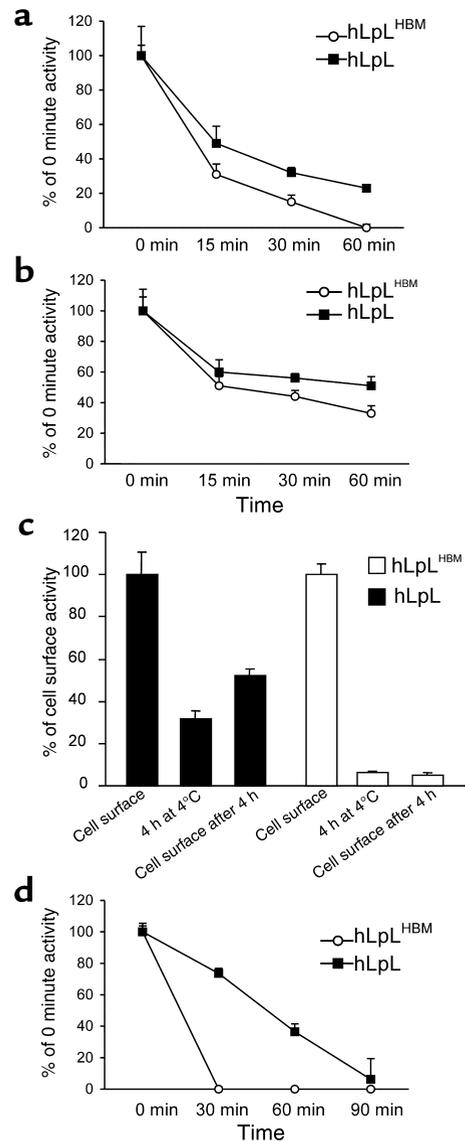
$n = 5$, and 1.26 ± 0.1 mmol FFA/l in hLpL/LpL0, $n = 4$). These fatty acids are mainly derived from adipose tissue stores and not circulating lipoproteins. In contrast, significant differences between these two genotypes were found in the same mice in the postprandial state at 1 and 2 hours (Figure 5). The postprandial TG was also significantly different; 1 hour after gavage TG was 2.2-fold higher in hLpL[HBM]/LpL0 ($P < 0.02$), at the 2-hour time point the elevation was 4.2-fold ($P < 0.02$) and 3 hours postprandial the TG was still 1.4-fold higher ($P < 0.02$) in these mice. Fasting as well as postprandial FFA did not differ in LpL1 littermates of both types of mice ($n = 5$ each, data not shown). Therefore, although hLpL-expressing mice removed TG more efficiently, suggesting more active lipolysis, hLpL[HBM] mice had more fatty acids in their bloodstream. One explanation for these results is that more chylomicron lipolysis occurred away from the microvascular bed in muscle of hLpL[HBM]/LpL0 mice. This might have led to less uptake of the newly generated FFAs by muscle tissue.

Metabolism of intravenously injected rat chylomicrons, Intralipid and palmitate. Rat chylomicrons with (^{3}H)fatty acid labeled TG and (^{14}C)cholesterol were used to assess plasma lipoprotein turnover, generation of FFAs within the circulation, and organ lipid uptake. As shown in Figure 6a, the fractional catabolic rate of these particles (^{3}H-label) in hLpL[HBM]/LpL0 was significantly slower than that of hLpL/LpL0 mice (0.14 ± 0.02 vs. 0.19 ± 0.01 pools/min, $P < 0.01$). However, significantly more fatty acids were generated in hLpL[HBM]/LpL0 ($4{,}466 \pm 1{,}275$ dpm/ml plasma vs. $1{,}180 \pm 1{,}278$ dpm/ml plasma in hLpL/LpL0, $P < 0.01$, Figure 6b). More of each label was found in muscles of hLpL/LpL0 and less in the kidneys (Figure 6c). This suggested that hLpL increased uptake of both newly generated fatty acids and core lipids. This was confirmed by assessing the uptake of Intralipid. Over 99% of the label was cleared after 10 minutes after an intravenous injection in the two groups of mice. In skeletal muscle there was more than 1.5-fold more fatty acid and cholesteryl ether uptake in hLpL/LpL0 compared with hLpL[HBM]/LpL0 mice ($P \leq 0.02$). Interesting-

ly, kidneys of hLpL[HBM]/LpL0 mice accumulated 35% more fatty acids ($P = 0.05$) and 50% more cholesteryl ether ($P < 0.02$) than kidneys of hLpL/LpL0 mice. This may have resulted from a greater amount of hLpL in kidneys from hLpL[HBM]/LpL0 mice (377 ± 42 versus 221 ± 34 ng/100 mg tissue, $n = 4$, $P = 0.001$). Therefore, the expression of the hLpL[HBM] transgene on the LpL0 background leads to more FFAs in plasma, less lipid uptake in skeletal muscle, and more uptake in kidneys than overexpression of nonmutated hLpL.

As shown in Figure 6d, the plasma clearance of (^{3}H)palmitate was similar in the two types of mice. By 15 seconds after injection approximately 75% of the injected dose was cleared from the circulation ($74.3 \pm 5.9\%$ in hLpL[HBM]/LpL0 and $75.6 \pm 6.6\%$ in hLpL/LpL0). Two or three minutes after injection approximately 98% of the injected dose had been removed from the bloodstream of mice with both genotypes. Therefore, differences seen in postprandial FFA and in tissue lipid uptake from Intralipid emulsion in these mice were not due to dif-
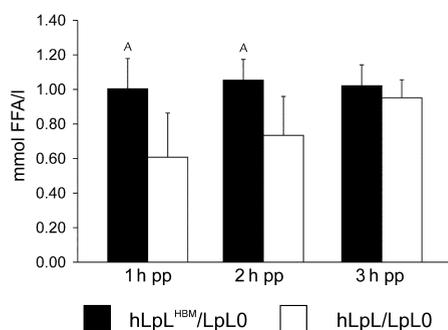
Figure 5

Postprandial FFAs. After 8 hours of daytime fasting, a gavage with 100 μl corn oil was performed. Blood was withdrawn from the retroorbital plexus and plasma was assayed for FFA at the indicated time points. [A]$P < 0.03$. pp, postprandial.

histochemical analysis on fixed and frozen sections as well as ultrastructural investigations on hand of semi- and ultrathin sections of Epon-embedded samples by electron microscopy revealed any evidence for a lipolysis-induced myopathy comparable to that with overexpression of hLpL.

Discussion

These studies were performed to define the regions of LpL required for its high affinity binding to HSPGs and to define the importance of HSPG binding in LpL metabolism and physiological actions. Several basic amino acids in LpL were mutated and both in vitro and in vivo studies were performed. Our experiments showed the following: (a) The carboxyl-terminal region was important for LpL binding to heparin, and cells, (b) hLpL[HBM] was found in the bloodstream in preheparin blood, suggesting that endothelial HSPG binding was defective in vivo, (c) abnormal heparin binding still allowed production of active LpL, although that LpL had reduced stability, (d) hLpL[HBM] transgene produced active LpL in vivo and prevented the neonatal demise of LpL knockout mice, (e) the hLpL[HBM] transgene led to greater amounts of postprandial FFAs, suggesting that more lipolysis was occurring in the bloodstream rather that along the lumen of capillary endothelial cells, and (f) compared with mice expressing nonmutated hLpL,
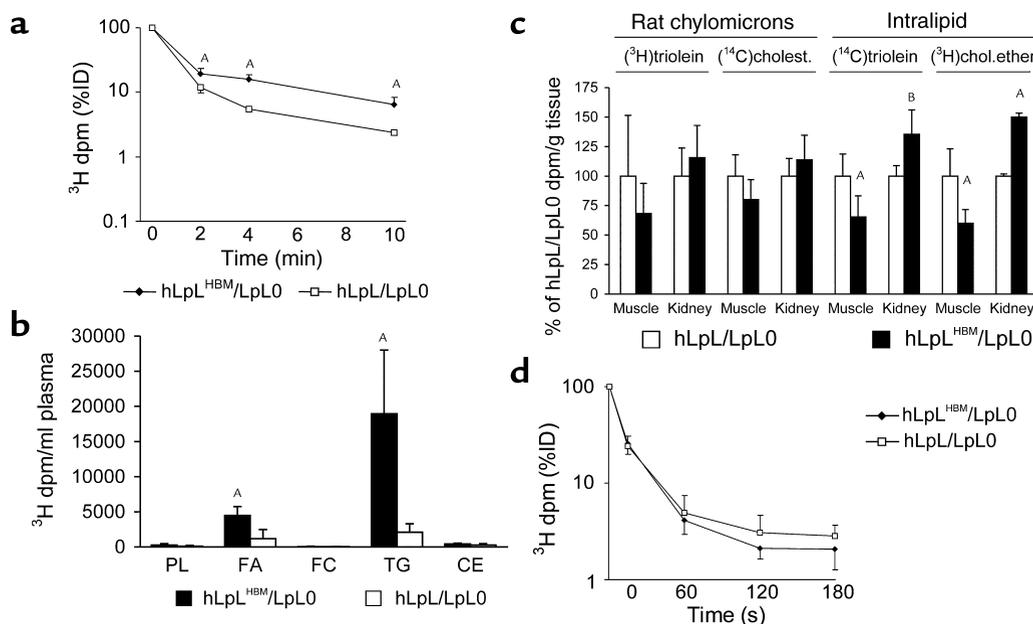
ferences in the mice's capability to remove fatty acids from the circulation.

Muscle histology. A final method to assess lipid metabolism of muscle was employed. Previous studies had shown that expression of hLpL on the wild-type background at levels similar to those of the hLpL[HBM] transgenic mice led to fatty acid–induced myopathic changes (20). Neither light microscopic analysis of routinely stained paraffin sections (Figure 7) nor further

Figure 6

Rat chylomicron, Intralipid, and palmitate turnover studies. (**a**) Rat chylomicrons were labeled in vivo with (^{3}H)triolein, collected as described in Methods, and injected into the tail vein of hLpL[HBM]/LpL0 (*n* = 5) and hLpL/LpL0 (*n* = 5) male mice. Blood was collected 2, 4, and 10 minutes after injection. Total plasma volume was calculated as 2.75% of body weight. Data are expressed as percentage of injected dose (ID). (**b**) A 100 μl aliquot of the plasma from the 10-minute time point was lipid extracted and separated by TLC. The bars show the ^{3}H dpm per ml plasma for the indicated lipid. (PL, phospholipids; FA, fatty acids; FC, free cholesterol; CE, cholesterol esters). (**c**) Ten minutes after injection of labeled rat chylomicrons or Intralipid (injected into four hLpL[HBM]/LpL0 and four hLpL/LpL0 female mice), the mice were perfused with PBS and the indicated organs were taken out. Lipids were extracted with chloroform-methanol 2:1 (42) and counted. The data are expressed as percentage of hLpL/LpL0 dpm/g tissue. (**d**) (^{3}H)palmitate was complexed to fatty acid–free BSA and injected into four hLpL[HBM]/LpL0 and four hLpL/LpL0 male mice. Mice were bled at the indicated time points. Data are expressed as percentage of injected dose. [A]$P ≤ 0.02$; [B]$P ≤ 0.05$. Cholest., cholesterol; chol.ether, cholesterol ether.
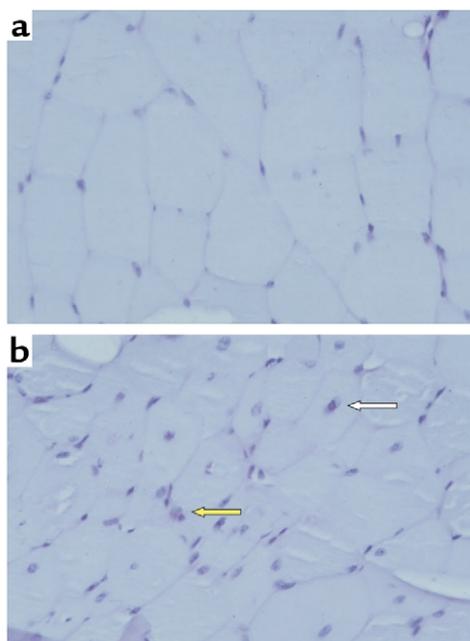
Figure 7
Histological examination of muscle from hLpL[HMB] transgenic mice. Periodic-acid Schiff–stained (PAS-stained) sections of quadriceps muscles of an 8-month-old female hLpL[HBM]/LpL2 mouse. In most areas completely normal histology was found (**a**). Only in small areas of hLpL[HBM]/LpL2 quadriceps some discrete, nonspecific myopathic alterations including more frequently centralized nuclei, a low number of in part atrophic muscle fibers, and some lipocytes in the endomysium could be found. Rarely a slight increase in glycogen storage could be detected (**b**). Compared with mice expressing high levels of hLpL, these findings are minimal. White arrow shows centralized nucleus, yellow arrow shows glycogen.

muscle expression of hLpL[HBM] led to less uptake of plasma lipids into muscle and, surprisingly, more lipid uptake in the kidney.

Our first objective was to determine whether the carboxyl region of hLpL is important for HSPG binding in vitro and in vivo. Cells expressing the hLpL[HBM] produced active enzyme protein, but the association of that protein with heparin affinity gel was markedly impaired. Inactive hLpL[HBM] protein in the preheparin plasma of transgenic mice eluted from the gel at about 0.4 M NaCl. A second protein peak found in postheparin plasma eluted at 0.8–1 M NaCl. This presumably dimeric LpL was clearly defective in heparin binding compared with normal LpL that eluted at greater than 1.2 M NaCl. Thus, the carboxyl-terminal mutations led to production of a LpL molecule that was active and had normal interaction with plasma lipoproteins but was defective in heparin binding. Lookene et al. (19) investigated LpL heparin and HSPG binding properties using a Biacore system (Biacore AB, Uppsala, Sweden). They introduced mutations similar to ours (amino acids 403, 405, and 407 were changed to alanine) in an isolated COOH-terminal LpL domain (amino acids 313–448). Although this mutated LpL fragment had virtually no heparin binding, they concluded that the

contribution of the COOH-terminal domain to heparin binding of LpL is about 20–40%. We show that in vivo the mutation of the COOH-terminal heparin-binding region markedly reduces heparin binding with several significant alterations in LpL's physiologic actions. However, our mutations did not completely eliminate the enzyme's binding to HSPGs.

The observation that a large amount of hLpL[HBM] was in the preheparin plasma of the transgenic mice was most consistent with reduced ability of this protein to remain associated with its capillary endothelial HSPG-LpL binding site. This would allow the protein to enter the bloodstream without the usual infusion of heparin. Other studies confirmed that hLpL[HBM] dissociated from isolated skeletal muscle more readily. This undoubtedly led to some of the increased LpL protein found in preheparin plasma. At this time, we cannot definitely rule out that some of the preheparin hLpL[HBM] was also due to an accompanying defect in removal of circulating LpL from the bloodstream. It may well be that binding to HSPGs in the sinusoids of the liver is a prerequisite for uptake and degradation of LpL by hepatocytes, since heparin infusion will block LpL removal from the bloodstream (12).

LpL, like a number of other proteins including growth factors and clotting factors, has a high affinity binding to heparin. There may be a number of physiologic reasons for this, and our data suggest that LpL heparin binding modulates its enzymatic activity and localizes the enzyme to tissues that are most important for uptake of lipoprotein-derived fatty acids. Concomitant with defective HSPG binding was a reduction in the stability of LpL activity; this was likely to have resulted from conversion of dimeric LpL to monomers. Thus, a fundamental role of HSPG binding illustrated in this study is maintenance of protein quaternary structure.

The physiological repercussions of reduced LpL association with HSPGs were also studied. Although hLpL-[HBM] contained sufficient enzymatic activity to "rescue" LpL knockout mice, these animals were more hypertriglyceridemic than wild-type mice and mice expressing nonmutated hLpL. This suggested that the hLpL-[HBM]/LpL0 mice had a mild defect in TG lipolysis. Catabolism of both injected rat chylomicrons and postprandial TG was defective. Despite reduced lipolysis, plasma FFAs were higher in hLpL[HBM]/LpL0 mice. Since the clearance of labeled palmitate from the bloodstream was not delayed in these mice, this observation is most consistent with hydrolysis of TG in the circulating blood rather than in close proximity to the vascular endothelium in capillary beds. This presumably occurred during the short period of time between the release of the active enzyme from the endothelial surface and its inactivation.

hLpL[HBM]/LpL0 and hLpL/LpL0 mice were compared to determine the effects of defective HSPG binding on tissue lipid uptake. Both histological studies and assessment of lipid turnover using a lipid emulsion showed less uptake of TG in the muscle of mice with

the defective enzyme. Of interest, hLpL[HBM] led to more uptake in the kidney that we hypothesize is due to the presence of more LpL protein in that organ.

Studies of the structure-function relationships of complex proteins have several limitations: (a) the mutated region could affect a number of processes in addition to the one being investigated, and (b) multiple regions may be required for several biochemical interactions. Mutations in the carboxyl-terminal domain have several effects on LpL in addition to altering its heparin affinity. One region required for LpL receptor–related protein (LRP) binding and presumably other members of this family of receptors includes amino acids 404–430 (36), a region that includes the carboxyl terminal heparin-binding domain. It is, however, unlikely that defective LpL binding to LRP would alter LpL dissociation from heparin or muscle. Moreover, LpL-LRP interaction is unlikely to explain why the hLpL[HBM] leads to more fatty acids in plasma after an intravenous or oral fat load. A number of other mutations in LpL clearly affect its ability to bind to heparin (13–15). Thus, high affinity heparin binding is likely to require a number of LpL-heparin interactions. Although these sites could also be studied in vivo, our overall objective was to define the physiological significance of the major heparin-binding domain of LpL.

Understanding LpL-proteoglycan interactions may explain the regulation of LpL under physiologic and pathophysiologic conditions. LpL regulation is complex and much of the variation of LpL activity occurs at a posttranslational level. Decreases in LpL activity in adipose tissue during fasting (37, 38) and with diabetes (39) are not associated with reductions in LpL immunoreactive protein. Thus, it is likely that the dimeric LpL protein becomes inactivated; our data suggest that dissociation from proteoglycan binding sites on the endothelium, adipocytes, or interstitial matrix is likely to be responsible for this protein inactivation. A second example of the importance of LpL-proteoglycan interaction is seen in the unusual patients with autoimmune syndromes leading to antibodies to heparin (40, 41). When these antibodies bind to HSPGs they presumably block access of LpL to these sites; the clinical presentation is severe hyperchylomicronemia due to lack of LpL-mediated TG hydrolysis and lack of LpL activity in the postheparin blood. Presumably the inability to bind to cell surface proteoglycans leads to rapid inactivation of LpL and an LpL-deficient phenotype.

In summary, we provide evidence that the carboxyl-terminal region of LpL is important for its association with HSPGs. Although the mutated region has several basic amino acids, it does not contain a classical heparin-binding motif. The physiological importance of defective heparin binding was demonstrated in genetically modified mice that only express the mutated LpL. Heparin binding via this carboxyl-terminal region is required to maintain LpL activity and probably prevents the conversion of the enzyme into inactive monomeric subunits. In addition, we show that optimal LpL binding to heparin is required to localize LpL to proteoglycan-rich cell membranes, leading to efficient targeting of newly created fatty acids to tissues. These studies are the first to our knowledge to assess the role of heparin binding in in vivo physiology and provide an approach to assess the importance of protein-proteoglycan interactions in other systems. Such interactions are critical for a number of other proteins including growth factors.

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