Endothelial bound lipoprotein lipase (LpL) depletion in hypoalbuminemia results from decreased endothelial binding, not decreased secretion

GC Shearer[1,2] and GA Kaysen[1,2]

[1]Department of Veterans Affairs Northern California Health Care System, Mather, California, USA and [2]Division of Nephrology, Department of Medicine UC Davis, Davis, California, USA

Hypertriglyceridemia in nephrotic (NS) and Nagase analbuminemic rats (Analb) results from reduced triglyceride clearance. NS and Analb have reduced or absent albumin, reduced plasma oncotic pressure (π), but Analb lack proteinuria. The heparin releasable lipoprotein lipase (LpL) pool in both models is greatly reduced, suggesting reduced LpL is related to low albumin or π and not proteinuria. To determine the cause of endothelial LpL reduction, we studied effectors of endothelial LpL (eLpL) levels from gene expression, to delivery and endothelial binding. eLpL was measured as heparin releasable activity. eLpL and secretion rate was measured in isolated hearts perfused with heparin. mRNA levels were measured in rat hearts by kinetic RT-PCR. Finally, binding of ^{125}I-LpL by competition assays rat endothelial cells measured serum-induced changes in affinity. eLpL *in vivo* was reduced in nephrotic and Analb rats. While the eLpL pool was reduced in isolated perfused hearts, neither LpL secretion by isolated hearts nor myocardial mRNA was reduced in NS or Analb. Binding of LpL to RAEC preincubated with serum from either NS or Analb was reduced compared to control. LpL mRNA levels and release rate was not altered in hearts from NS rats, while eLpL is depleted, suggesting that reduced eLpL in NS is not the result of reduced delivery. The finding that NS serum alters LpL binding to RAEC suggests LpL depletion results from decreased binding rather than defective delivery. This in turn is a consequence of reduced serum albumin or π but does not require proteinuria.

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Correspondence: *GC Shearer, Department of Veteran's Affairs – VANCHCH, c/o IM: Nephrology, University of California Davis, Genomics and Biomedical Sciences Building, Room 6300, Davis, California 95616, USA.*
E-mail: gcshearer@ucdavis.edu

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Hypertriglyceridemia occurs commonly in the nephrotic syndrome. While both increased secretion[1] and decreased catabolism[2] of triglyceride-rich lipoproteins (TRL),[3] including[4,5] chylomicrons and very low-density lipoproteins (VLDL), have been reported, recent evidence suggests reduced TRL catabolism is most important in establishing their increased levels.[6–9]

We have demonstrated that proteinuria alters the structure and function of both high-density lipoprotein and VLDL contributing to decreased VLDL clearance.[10,11] However, an identical defect in chylomicrons uptake by perfused hearts isolated from either analbuminemic (Analb) or nephrotic rats (NS) rats suggests that an endothelial defect arising from hypoalbuminemia even in the absence of proteinuria also contributes to decreased lipoprotein clearance in both models.[10]

Both the fractional and absolute rate of lipolysis of exogenously administered chylomicrons is reduced in rats with an experimental model of the nephrotic syndrome.[2] The catabolic defect[8,12] persists in isolated tissue (heart),[9] suggesting that a change in the endothelium contributes at least in part to the reduced TRL catabolism. The rate-limiting enzyme responsible for TRL triglyceride (TG) lipolysis, lipoprotein lipase (LpL), is associated with the vascular endothelium. Endothelial cells do not produce LpL directly. Instead, it is produced by mesenchymal tissue – such as myocardial and skeletal muscle, adipose tissue, and mononuclear cells – and undergoes a process of translocation to the endothelium.[13] LpL is sorted in the Golgi and is directed to either exocytosis or to intracellular degradation. Since intracellular LpL reaches catalytic maturity as early as the endoplasmic reticulum,[14] nonsecreted LpL comprises tissue-associated LpL activity that is not heparin releasable, and confounds the use of total tissue LpL as marker for lipolytic capacity. After LpL is released from the synthesizing cell, it binds to heparan sulfate on the secreting cell, forming an interstitial LpL pool. This binding is important because LpL's association with heparan sulfate imparts stability to the enzyme.[15] It is then translocated from the interstitial pool to the vascular endothelium where it binds to heparan sulfate moieties on the vascular endothelium forming an endothelial

LpL pool. This endothelial bound pool is physiologically relevant because only this pool is capable of interaction with VLDL for lipolysis or internalization. Heparin can displace LpL from its attachment to heparan sulfate moieties on the vascular endothelium and release it from interstitial spaces due to LpL's higher affinity for heparin than for heparan sulfate. Both the endothelial and interstitial pool then form potential heparin releasable pools of LpL.

Total heparin releasable LpL has been reported to be reduced *in vivo* both in experimental models of the nephrotic syndrome[2,7,9,16–18] and in nephrotic patients.[19] These findings suggest that a reduction in the activity of the endothelial LpL pool plays a contributing role in reducing TRL clearance. The mechanism responsible for the reduced endothelial LpL pool is not established. Potential mechanisms include reduction in synthesis, secretion, transport, or binding of LpL to the endothelial surface. Additionally, it is unclear how the mechanism(s) responsible for LpL depletion is associated to the rest of the pathophysiology of the nephrotic syndrome.

It is not clear whether the LpL depletion results from proteinuria *per se*, or if it is a consequence of reduced albumin concentration or π. We studied LpL levels in two models of reduced albumin concentration, rats with hereditarily analbuminemia – the Nagase Analb rat and rats with hypoalbuminemia resulting from urinary protein loss (the nephrotic syndrome) to establish the effects of hypoalbuminemia on LpL in the presence and absence of urinary protein loss. Analb rats exhibit some aspects of nephrotic syndrome although they lack proteinuria, including increased levels of fibrinogen, macroglobulins, transferrin, apolipoprotein A (apoA)-I[20–23] as well as elevated plasma TG and cholesterol and reduced heparin releasable LpL.[8,16] Thus, Analb rats serve as a model of nephrotic syndrome in the absence of proteinuria. TG levels are significantly less in Analb than in nephrotic animals consistent with a partial defect in lipoprotein metabolism imparted by low albumin levels alone in the absence of proteinuria.

In order to determine the mechanism of LpL depletion in the nephrotic syndrome, we measured heparin releasable LpL from intact nephrotic animals, having both reduced albumin levels and proteinuria and Analb animals, having isolated hypoalbuminemia in the absence of urinary protein loss as well as LpL heparin inducible release, delivery and mRNA levels in isolated hearts from these same groups of animals. We also established the effect of serum from nephrotic and Analb animals on the binding of LpL to endothelial cells.

RESULTS

Serum TG levels were significantly increased in NS rats (257 ± 30 mg/dl in comparison to Analb (120 ± 17) and control (30 ± 15 mg/dl). All three groups were significantly different than one another ($P < 0.05$).

Heparin releasable LpL

In both NS and Analb rats, total body releasable (*in vivo*) heparin releasable LpL activity was reduced to 14.2 ± 4.9 and $2.8 \pm 6.2\%$, respectively, of controls (Figure 1). Within the nephrotic group itself, heparin releasable LpL activity was a significant predictor of serum TG levels and accounted for 88% of the variability in serum TG levels ($r^2 = 0.881$, $P = 0.018$), but heparin releasable LpL activity did not correlate with serum TG in either Analb or control.

Myocardial LpL secretion

Similar to what was observed in the whole body, the release of LpL from typical rat hearts perfused continuously with heparin is shown in Figure 2. We found that most control hearts had an initial peak at 1–3 min post-heparin, and an additional peak at 3–4 min. The additional peak was fused with the first peak in three control and five NS rats and all six Analb rats. In all rats, LpL activity reached a stable baseline by 12 min and no subsequent changes in released activity were observed. We corrected LpL activity for perfusion rate and heart weight to determine LpL secretion rate.

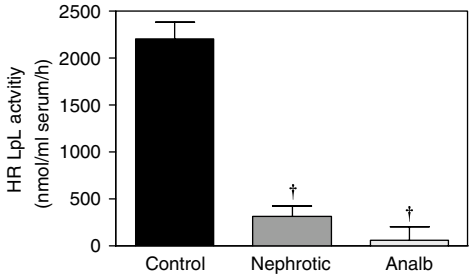

Figure 1 | *In vivo* heparin releasable LpL in control, NS, and Analb rats. Serum was obtained prior after a 12-h fast followed by injection of 50 U/kg heparin was injected into the femoral vein. The animal was exsanguinated 5 min later and LpL activity was measured. The mean of LpL activity in control, NS, and Analb rats are shown $\pm$ s.e.m. $^{\dagger}P < 0.0001$.

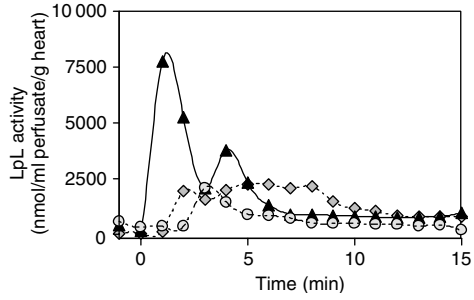

Figure 2 | LpL pools in perfused hearts. Release of LpL activity into perfusate by control, NS, and Analb hearts upon perfusion with 5 U/ml heparin. Zero minutes represent the time of heparin addition to the perfusate. LpL activity in control perfusate (▲) has an initial peak representing LpL bound to heparan sulfate glycosaminoglycans on vascular endothelial cells, a second smaller peak representing LpL bound to heparan sulfate on myocardial cells, and a final stable baseline representing the endogenous rate of LpL secretion by myocardial cells. The other lines represent release by a typical nephrotic (◊) or Analb (○) heart. Noticeably, there was either a very small or a nonexistent initial peak in hearts from NS and Analb animals. The second peak was fused with the first in three control, and was indistinguishable in all NS and Analb hearts.

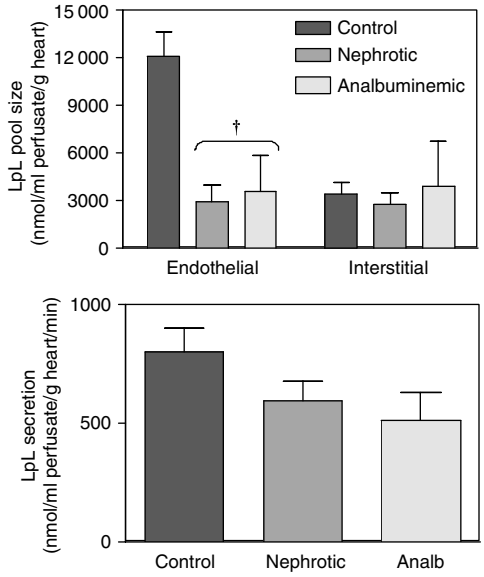

Figure 3 | Quantitation of LpL peaks and secretion rate. For each heart, the area under the first and second peak was determined and is represented in the first graph: control, NS, and Analb. The endogenous LpL secretion is represented in the second graph. The data shown are mean ± s.e.m. $^{\dagger}P < 0.0001$.

Bagby attributes the two initial peaks and the stable baseline to 1: endothelial bound LpL, 2: vesicular or interstitial LpL, and 3: the endogenous rate of LpL synthesis.[24] More recent research indicates that Golgi-LpL not directed to lysozomal degradation is exocytosed and binds to heparan sulfate moieties on the cell surface.[13,25] This suggests that the second LpL peak is indicative of the interstitial LpL pool and the final, stable release indicates the rate of cellular secretion, to the interstitial and endothelial pools.

The quantitation of these pools is represented by the bar graphs in Figure 3. Figure 3a depicts quantitatively the endothelial and interstitial pool sizes while Figure 3b represents the cellular secretion rate. The specific endothelial associated LpL pool was reduced in NS rats to 23.9% of control (control = 12061 ± 1559; NS = 2885 ± 1077; Analb = 3565 ± 2277 nmol/ml perfusate/g heart; $P < 0.05$). No differences were detected in the interstitial pool (control = 3417 ± 686; NS = 2714 ± 748; Analb = 3884 ± 2836), or in the cellular secretion rates (control = 797 ± 102; NS = 593 ± 79; Analb = 509 ± 117).

LpL mRNA levels in heart tissue

We found no differences in the levels of LpL mRNA in NS rats compared to controls (Figure 4). Levels of LpL mRNA in NS rats tended to be only slightly reduced compared to control (Neph = 0.95 ± 0.23, Analb = 0.71 ± 0.03, Con = 1.04 ± 0.17 LpL/GA3PDH).

Binding of exogenous ^{125}I-LpL to cultured aortic endothelial cells

The binding of exogenous LpL to cultured rat aortic endothelial cells (RAEC) was significantly reduced in cells

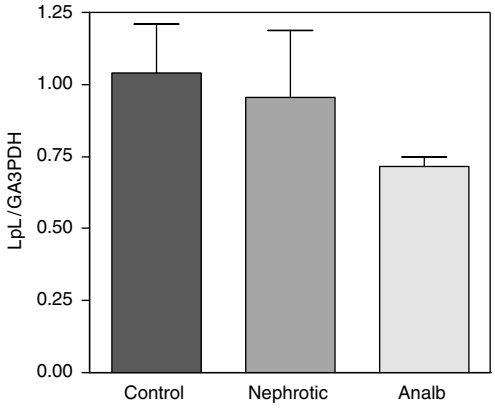

Figure 4 | Expression of myocardial LpL by quantitative PCR. Levels of mRNA in control, NS, and Analb hearts were measured by quantitative PCR after perfusion. The ratio of LpL to GA3PDH was determined. No differences in the relative levels of LpL mRNA were observed.

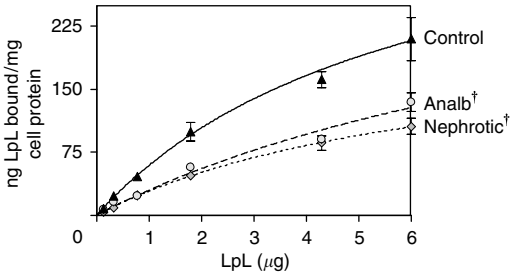

Figure 5 | Binding of exogenous ^{125}I-LpL to serum treated RAEC. Binding of LpL was measured after treatment of cells with media containing 20% serum from control, NS, or Analb animals. Cells were grown to confluence with 20% fetal bovine serum and treated with serum experimental serum for 18 h prior to determination of LpL binding. Specific LpL binding was assessed by competition of ^{125}I-LpL with unlabeled LpL.

treated with serum from nephrotic animals compared to serum from control (Figure 5) ($P < 0.0001$). We tested whether this effect of nephrotic syndrome persisted in the absence of proteinuria and of severe hypertriglyceridemia. Binding of LpL to RAEC treated with serum from Analb was also reduced compared to control ($P < 0.0001$) and was not different from treatment with nephrotic serum.

DISCUSSION

The reduction of LpL in NS rats is a consequence of a specific reduction of LpL at the vascular endothelial surface. This reduction is not the result of decreased synthesis or release of LpL from mesenchymal tissue since interstitial LpL levels are normal, interstitial cell LpL secretion is normal, and LpL mRNA levels are normal in rat heart tissue regardless of animal origin. Additionally we previously demonstrated that residual LpL activity in cardiac muscle in NS and normal rats is the same following perfusion with heparin.[8] Thus, the reduction in cardiac LpL pool is confined to heparin labile LpL, the fraction bound to endothelial and interstitial cell glycosaminoglycans. The bulk of LpL clearly resides in

noncardiac tissues, including skeletal muscle and adipose tissue. While the heart might not be quantitatively representative of all LpL pools, total body heparin releasable LpL was decreased to at least a similar extent to what we observed in the heart. Thus, it is likely that similar changes in LpL release occur in other tissues. Glycosaminoglycans, including both heparin and heparan sulfate, bind to and stabilize LpL.[15,25] Since we perfused isolated hearts with heparin, it is possible that we provided a more protective environment than exists *in vivo* and failed to detect loss of LpL activity during the translocation from cardiac myocytes and delivery to capillary endothelial cells. Our observation that serum from hypoalbuminemic NS or Analb animals decreases LpL binding to endothelial cells makes such a hypothesis unnecessary. We propose that the decrease in endothelial bound LpL is the result at least in part of decreased LpL binding to endothelial cells, and furthermore that these alterations are a consequence of hypoalbuminemia or reduced plasma oncotic pressure (π) and does not require proteinuria.

We found that heparin releasable LpL activity is a significant predictor of serum TG in NS animals, suggesting a critical role for LpL in TRL catabolism in the NS. While heparin releasable LpL predicted serum TG in NS it did not do so in normal rats; however, LpL is present in great excess in normal animals. What is of interest is that TG levels are only slightly increased in Analb even though LpL is reduced to the same extent in these animals as it is in nephrotic animals. We have hypothesized previously that proteinuria confers an additional defect on VLDL structure not resulting from decreased albumin levels alone, rendering its binding to endothelial bound lipase to be reduced.

Our current observations provide a link between altered lipoprotein metabolism that is independent of proteinuria, and insufficient in the absence of altered lipoprotein structure, to cause great increases in TG levels. Nevertheless, hypoalbuminemia in these models induces one component of reduced lipoprotein catabolism that is independent of proteinuria. When this component is linked to altered lipoprotein structure caused by proteinuria then significant TG levels result.

Proteinuric rats have a combined defect that results in delayed clearance of TRL.[10,11] Proteinuria alters VLDL structure such that binding to LpL is impaired. Clearance of TRL therefore is of increased dependence upon a rich endothelial bound LpL pool. Thus in NS, the level of endothelial bound LpL clearly presents a dose dependent barrier to TRL clearance.

The LpL depletion in NS rats is unlikely to be due to changes in gene expression. We found no difference in LpL mRNA levels in hearts from rats with nephrotic syndrome induced by adriamycin. Liang and Vaziri[26] did note a difference in LpL mRNA in hearts obtained from rats rendered nephrotic with puromycin aminonucleoside, but not in other tissues. They hypothesized the presence of a defect distal to mRNA processing.

Endothelial LpL levels are not normally regulated at the level of LpL gene expression, and our findings also indicate that changes in expression are not necessary for depletion of endothelial LpL pools. If reduced expression of the LpL gene were the only mechanism responsible for its depletion on the vascular endothelium, one would anticipate that release of this protein as well as its level in all tissue compartments would be reduced. Our previous observation that residual tissue bound LpL was the same in hearts from nephrotic and control animals while heparin releasable Lpl was significantly reduced in hearts from nephrotic animals is consistent with this hypothesis.[8]

We next established that the depleted LpL pool that is depleted is the endothelial pool, and that the interstitial pool is not depleted, suggesting that LpL is both synthesized and released by the heart in nephrotic and control animals at similar rates. The principal LpL pool depleted is that bound to the vascular endothelium, suggesting that there is either a defect in its binding to the vascular endothelium in the presence of low albumin, or that LpL once bound is rapidly destroyed.

We tested the hypothesis that LpL binding to the vascular endothelium is reduced by serum from animals with low albumin levels regardless of whether or not proteinuria was present. Specifically, endothelial cells pretreated with NS or Analb serum have reduced binding to the vascular endothelium.

There are a number of possible mechanisms for this reduced LpL binding. First, LpL's association with heparan sulfate stabilizes the enzyme.[15] LpL binds to specific glycosylation sequences, which may change or whose number may be reduced in the nephrotic syndrome. Evidence for proteoglycan changes in nephrotic syndrome are abundant.[27–29] Alternatively, Pang *et al.*[30] have identified an N-terminal fragment of apoB that is synthesized in endothelial cells and is released to the lumen surface where it increases LpL binding. Finally, Pillarisetti *et al.*[15] also report that circulating levels of lyso-phosphatidyl choline (LPC) alter the binding affinity of LpL to heparan sulfate proteoglycans. Sasaki and Goldberg[31] demonstrated that 3% albumin prevented a defect in the binding of LpL to heparan sulfate proteoglycan caused by LPC. However, Joles[32,33] observed that free LPC levels were not increased in Analb making such a mechanism difficult to invoke as a cause of the changes that we observed. Nevertheless, we have not directly established the mechanism whereby the presence or absence of albumin in serum causes reduced lipase on an endothelial surface. The presence of a substance that is normally bound by albumin that inhibits lipase binding is clearly one potential mechanism. It is possible that that reduced LpL or apoE reduces heparan sulfate proteoglycan since both these proteins have been demonstrated to enhance proteoglycan production.[34] The particular dynamics of our findings limit the expectation that reduced apoE or LpL are the source and altered heparan sulfate proteoglycan is the consequence. LpL secretion is not reduced. Analb rats have normal lipoprotein apoE content, unlike proteinuric rats whose lipoproteins are apoE depleted.

This is true even of mildly proteinuric animals with normal plasma lipids.[35] Changes in any of these parameters in the nephrotic syndrome could result in defective LpL binding and/or stability at the vascular endothelium.

MATERIALS AND METHODS

Animals

Studies were approved by the Animal Review Committee at the University of California Davis and the Department of Veterans Affairs. Rats were all male Sprague–Dawley obtained from Simenson Farms (Hayward, CA, USA). They were kept in 24 h light/dark rooms and fed *ad libitum*. Nephrotic syndrome was induced at 5 weeks of age by tail vein injection of 5 mg/kg adriamycin (Adriamycin RDF: Pharmacia & Upjohn, Milan, Italy). Urinary albumin excretion was measured 4 weeks after injection with adriamycin and rats with albuminuria of >350 mg/day were selected. In each experiment, rats were anesthetized with an intraperitoneal injection of 0.75 g/kg (control) or 0.40 g/kg (nephrotic) sodium pentobarbital. No heparin was used.

Quantitative reverse transcriptase-polmerase chain reaction

RNA was isolated from hearts of three control, three NS, and three Analb rats. The heart was immediately frozen in liquid nitrogen and stored at 70°C until extracted. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol and dissolved in DEPC-treated water. RNA (1 μg) was incubated for 30 min with 1 U DNase I (Invitrogen) to digest contaminating genomic DNA, then reversed transcribed using 200 U M-MLV reverse transcriptase (Invitrogen) and 0.5 μg oligo-dT primer (Invitrogen) in a buffer containing 1 mM dNTP's (Promega, Madison, WI, USA), 3 mM $MgCl_2$, and 7 U RNase inhibitor (Invitrogen). Quantitative polymerase chain reaction (PCR) was performed using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Invitrogen) for fluorescent detection of the double-stranded PCR products. Each 50 μl reaction contained 10 pmol of each primer, 200 μM dNTP's, 1.25 U HotStartTaq DNA polymerase (Qiagen, Valencia, CA, USA), 5 μl SYBR Green I diluted 1:1000 in DMSO, 1.5 mM $MgCl_2$, and buffer supplied with the DNA polymerase. The primer sequences for LpL were 5′-GCA GGA AGT CTG ACC AAC AAG-3′(forward) and 5′-CTT CAC CAG CTG GTC CAC AT-3′ (reverse). The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CTG GAG AAA CCT GCC AAG TAT GAT-3′ (forward) and 5′TTC TTA CTC CTT GGA GGC CAT GTA-3′(reverse). The relative degree of LpL expression in treated animals vs controls was determined by comparing the cycle number at which a specified threshold fluorescence emission from the 294 bp LpL PCR product was achieved, C_t^{LpL}, normalized to the C_t^{GAPDH} of the 267 bp GAPDH PCR product for each cDNA sample. The relative level of LpL expression is calculated by $2^{-(C_t\text{LpL}-C_t\text{GAPDH})\,\text{treated}/(C_t\text{LpL}-C_t\text{GAPDH})\text{control}}$.

Purification of LpL

LpL was purified using the methods of Liu *et al.*[36] Briefly, raw skim milk, 0.4 M NaCl was incubated at 4°C over night with heparin-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Heparin sepharose was loaded onto into an XK-26 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and rinsed with 20 mM Tris, pH 7.4, 20% glycerol, 0.5 M NaCl buffer followed by 0.75 M NaCl until the absorbance at 280 nm reached background. LpL was eluted with 1.5 M NaCl and the fractions with an optical density of $\geqslant$(0.200 AU) at 280 nm were subsequently analyzed by SDS-PAGE. Fractions containing a single darkly staining band at 55 kDa were pooled.

LpL activity

LpL activity was assessed using a protocol adapted from Krauss *et al.*[37] In total, 10 μCi of ^{3}H-triolein, 1.5 mg phosphatidyl choline, and 37.5 mg triolein in chloroform solution were combined in a $50 \times 16\,mm^2$ tube and dried completely under N_2. A measure of 2.8 ml substrate buffer (0.2 M Tris, 0.150 M NaCl, pH 8.6), 1.2 ml 10% fatty acid free bovine serum albumin in substrate buffer, 0.25 ml 1% Triton X-100 substrate buffer, and 0.25 ml fasting heat inactivated human plasma were added to the tube. The solution was then sonicated on ice using a Branson Sonifier 250 for 5 min at power output 5 and 50% duty.

Emulsion (0.1 ml) was then added to 0.4 ml sample or to sample diluted with substrate buffer. The samples were incubated for 30 min at 37°C. The reaction was stopped by the addition of 1.62 ml chloroform/MeOH/heptane (1.25:1.41:1.00) to each tube. The tubes were vortexed, and 0.50 ml of 0.4 M boric acid/0.4 M K_2CO_3, pH 10.0 was added. The samples were vortexed again for 15 s and then centrifuged for 10 min at 1000 *g* to separate phases. One milliliter of the top phase was counted in liquid scintillation fluid on a Beckman Scintillation counter. Emulsion (0.1 ml) was counted as standard for oleic acid release and an LpL standard curve was generated using purified LpL. The curve was linear with respect to LpL mass. Samples from *in vivo* measurements of heparin releasable LpL also contain hepatic lipase. We used 1.0 M NaCl to inhibit LpL activity and plasma LpL was measured as the difference between lipase activity in the absence and in the presence of 1.0 M NaCl.

In vivo heparin releasable LpL activity

In order to establish the effect of reduced serum albumin on total heparin releasable LpL, five nephrotic, five Analb, and five control animals were injected intravenously with 50 U/kg heparin. After 5 min, the rats were exsanguinated by aortic puncture. Blood was collected into tubes containing ethylenediaminetetraacetic acid and the plasma was collected and stored at −70°C until assayed for LpL activity.

Perfusion of hearts

Eight control, six NS, and six Analb animals were used. The hearts were excised and perfused retrograde with Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 1% albumin and 4.0 g/l glucose by the method of Langendorff. The perfusate and the perfusate chamber were kept at 37°C. Flow was maintained at 7 ml/min/g heart and the perfusate was not recirculated. The heart rate was monitored every 10 min for deterioration of heart rate. Hearts with declining heart rates were rejected. For the first 5 min, residual blood was washed out. Afterwards, the perfusate pool was switched to one containing perfusate buffer + 5 U/ml heparin, an amount which causes maximal release of LpL.[38] Perfusate fractions were collected from 2 min prior to heparin perfusion until 15-min postheparin perfusion. After 15 min, perfusate was collected at 5 min intervals for 30 min. The fractions were stored at −70°C until assayed for LpL activity.

Cells

A primary culture of RAEC was obtained by the method of McGuire and Orkin[39] from a control rat. The thoracic aorta was gently removed, cleaned, and sliced into 2–3 mm sections under sterile

conditions. These sections were laid onto a layer of Matrigel (Becton-Dickinson, Bedford, MA, USA) in a 24-well cluster plate, which had been preconditioned for 1 h with E-STIM (BD Biosciences, San Diego, CA, USA) endothelial cell growth medium (Becton-Dickinson) supplemented with epidermal growth factor and endothelial cell growth supplement. After 8 days of growth, the explants were removed and remaining cells were allowed to grow for an additional week whereupon they exhibited cobblestone morphology typical of endothelial cells. The cells were removed from the Matrigel by incubation with 2% dispase for 30 min and passed into a 25-cm^2 tissue culture flask coated with collagen I. The cells were thereafter grown on collagen I under the aforementioned growth supplement. Due to the presence of smooth muscle cells, the cells were trypsinized, and diluted for single-cell cloning by passage into 48-well cluster plates. A subclone staining negative for α-smooth muscle actin and positive for uptake of acetylated DiI-LDL was selected for further expansion and thereafter grown with Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, and 20 mM glycine.

LpL binding to cultured RAEC

Bovine LpL was iodinated by the method of McFarlane as modified by Helmkamp *et al.*[40] Iodinated LpL was dialyzed exhaustively with 20 mM Tris + 20% glycerol, pH 7.4 until the counts were 98% precipitable by trichloroacetic acid. For binding studies, RAEC at passage 12 were plated into 48-well plates and experiments were performed 1–2 days after reaching confluence. Serum from five control rats was pooled. In total, 10% dilution of this pool in DMEM was used to replace fetal bovine serum for 18 h prior to incubation with LpL after the cells had reached confluence. Similarly, pools of five NS or five Analb rats were used in the experimental assays.

Media containing DMEM, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid pH 7.4, and lipoprotein deficient fetal bovine serum was used to prepared appropriate concentrations of labeled and unlabeled LpL. Prior to assaying binding, some wells were incubated with 1.0 Sigma U/ml heparitinase (Seikagaku) for 1 h. To solutions containing 0.5 μg/ml ^{125}I-LpL, unlabeled LpL was added to achieve total LpL protein concentrations of between 0.5 and 6 μg/ml. Wells containing 5 U/ml heparin were used to assess nonspecific binding. Forty eight-well plates were removed from incubators and allowed to cool on ice for 30 min. LpL containing media (0.125 ml) was added to wells and each concentration was assayed in triplicate. Plates were gently shaken for 4 h at 4°C. The cells were then rinsed 3× with DMEM + 2 mg/ml albumin at 4°C, incubated 2× for 10 min with DMEM + 2 mg/ml albumin at 4°C, and rinsed twice times with DMEM at 4°C. Finally, the cells were dissolved in 0.25 ml 0.1 M NaOH and counted on a Packard gamma counter.

Statistical analysis

The data were analyzed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. Differences were determined using Student's *t*-test and correlation was determined using the least-squares method. Differences in slope were determined using an F-test to calculate the *P*-value. All data are reported mean ± s.e.m.

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