Lipoprotein lipase (LpL) affects low density lipoprotein (LDL) flux through vascular tissue: evidence that LpL increases LDL accumulation in vascular tissue

J. C. Rutledge[1] and I. J. Goldberg

Departments of Medicine, University of California, Davis, CA 95616 and Columbia University, College of Physicians and Surgeons, New York, NY 10032

Abstract A cardinal feature of the atherosclerotic lesion is increased low density lipoprotein (LDL) content of the arterial wall. Such increases in vascular wall LDL could result from either increased flux of circulating LDL across the arterial endothelial barrier or decreased efflux of LDL that has entered the vascular tissue. A number of studies have focused on factors that alter permeability of endothelial cell monolayers and intact blood vessels causing increased LDL influx. In contrast, the current studies were designed to test the hypothesis that lipoprotein lipase (LpL) increases LDL accumulation and decreases LDL efflux from vascular tissue. Frog mesenteric venular microvessels were cannulated and the rates of fluorescently labeled LDL accumulation (N/t) and efflux ($T_{½}$) were measured by quantitative fluorescence microscopy. When the vessels were perfused with a solution containing bovine milk LpL (10^{-5} g/ml) and human LDL (protein = 0.68 mg/ml), N/t was >15× greater than that of control vessels which were perfused with LDL alone. LpL addition did not change albumin permeability, suggesting that increased N/t was not related to changes in vessel permeability. Increased LDL accumulation within the vessel could have resulted from either an increase in LDL influx from the vessel lumen into the vascular tissue or a decrease in efflux of LDL. Therefore, LDL efflux from vascular tissue was determined by measuring the rate of decline in fluorescence intensity of control and LpL-treated vessels after washout of the vessel lumen with a clear, nonfluorescent solution. The half-life of fluorescence decay after LDL perfusions ($T_{½}$) was 4.2 ± 1.6 (SD) sec and 53.3 ± 15.5 sec after LpL (10^{-5} g/ml) was added to LDL indicating reduced efflux of LDL in LpL-treated vessels. Heparin prevents interaction of LpL with proteoglycans on and within the vascular tissue and in low concentration does not interfere with the enzymatic actions of LpL. Addition of heparin to solutions containing LDL and LpL almost completely eliminated the LpL-mediated increase in vascular tissue LDL accumulation. These results suggest that the increase in LDL accumulation requires the interaction of LpL or LpL-LDL complexes with vascular tissue proteoglycans. We hypothesize that LpL serves as a molecular bridge between LDL and proteoglycans of in vivo perfused blood vessels.—**Rutledge, J. C., and I. J. Goldberg.** Lipoprotein lipase (LpL) affects low density lipoprotein (LDL) flux through vascular tissue: evidence that LpL increases LDL accumulation in vascular tissue. *J. Lipid Res.* 1994. **35:** 1152–1160.

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Atherosclerosis is characterized by intracellular and extracellular accumulation within arterial walls of cholesterol and cholesterol ester that is derived from LDL. This accumulation of vascular wall LDL may result from greater influx of LDL from the circulation into the intima of vessels or from decreased efflux of LDL from the vascular wall. Theoretically, LDL influx can increase by several mechanisms: *1)* damage to endothelial cells, *2)* increased endothelial cell permeability, *3)* increased hydrostatic pressure, or *4)* higher plasma LDL concentration. Increased LDL influx and atherosclerosis may occur without obvious endothelial cell damage (1). One mechanism for this could be transient localized increases in endothelial cell permeability to several molecules of biological significance. Our previous studies have shown that LDL influx into microvascular walls is increased by modulators of vascular wall permeability such as calcium ionophores and histamine (2–4). Others have shown that macromolecular permeability of endothelial cell monolayers is increased by bacterial lipopolysaccharides (5), oleic acid (6), and the products of tobacco smoke (7). Furthermore, areas of arterial walls that have increased LDL accumulation have been positively correlated with increased vascular wall permeability in the rabbit aorta (8). Therefore, changes in endothelial cell permeability may initiate and/or potentiate atherogenesis.

In some situations accumulation of vascular wall LDL may be due to a defect in LDL efflux, without an increase

Abbreviations: Δ, change; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; FITC, fluorescein isothiocyanate; I_f, fluorescence intensity; I_{f0}, initial fluorescence intensity; I_{fmax}, maximal fluorescence intensity; LpL, lipoprotein lipase; LDL, low density lipoprotein; mM, millimolar; P_s, apparent permeability coefficient; N/t, rate of LDL accumulation; $T_{½}$, half-time of LDL efflux; TRITC, tetramethylrhodamine isothiocyanate; t, time.

[1]To whom correspondence should be addressed at: Division of Cardiovascular Medicine, T.B. 172, Bioletti Way, University of California, Davis, CA 95616.

in LDL influx. This would occur if LDL in the vascular wall did not return to the circulation or the lymph. Such a decrease in LDL efflux appears to occur in some models of atherosclerosis. Schwenke and Carew (9) reported that areas of atherosclerosis in cholesterol-fed rabbits had increased LDL retention, or decreased efflux, but had not increased LDL influx. Several distinct processes may lead to decreased LDL efflux. These include LDL binding to components of the vascular wall, LDL uptake by cells, and LDL alterations in size or charge that retard LDL efflux from the vascular wall. Native LDL binds poorly to matrix proteins such as proteoglycans; however, aggregation, oxidation, and alterations in LDL size and lipid composition will increase LDL association with proteoglycans (10–13).

Zilversmit (14) observed that atherosclerotic lesions contain increased lipoprotein lipase (LpL) activity. LpL is the rate-limiting enzyme required for hydrolysis of circulating triglyceride-rich lipoproteins. This enzyme interacts with chylomicrons and very low density lipoproteins while attached to the luminal side of endothelial cells. Recently, several groups of investigators have shown that LpL is synthesized by macrophage-derived foam cells and that LpL protein is found in foam cell-rich lesions (15, 16). LDL is known to accumulate in these same areas of atherosclerotic plaques (17, 18). Saxena et al. (6) recently hypothesized that LpL within vascular tissue, and not on the luminal endothelial surface, potentiates atherosclerosis by increasing LDL association with proteoglycans. LpL has lipid/lipoprotein and glycosaminoglycan binding domains (19–23) and, potentially, LpL could bind both to lipoproteins such as LDL and glycosaminoglycans of the vascular wall. If LpL increased LDL accumulation within the vascular tissue, it may do so by anchoring LDL to proteoglycans thereby decreasing its efflux. Several groups have now confirmed that LpL will increase LDL association with extracellular and cell surface proteoglycans (6, 24–27).

The current studies were designed to test the hypothesis that LpL increases LDL accumulation and decreases LDL efflux in an intact blood vessels. Because infusion or inhibition of LpL in the whole animal will lead to dramatic alterations in circulating lipoprotein concentrations, the studies described here were performed using in vivo perfused microvessels. In addition to providing additional support for the pathological role of LpL, our studies describe a new method for assessing LDL efflux from vessels.

MATERIALS AND METHODS

Materials

LpL was purified from fresh unpasteurized bovine milk using heparin-agarose affinity chromatography (Bio-Rad, Rockville, MD) and specific activity was determined as described previously (28). At the time of storage, LpL preparations had a specific activity of approximately 20–30 mmol free fatty acids/h per mg protein. The purified protein was stored at −70°C.

Bovine serum albumin was obtained from Sigma (St. Louis, MO, No. A4378) crystallized and lyophilized. Heparin sodium (Elkins-Sinn, Inc; Cherry Hill, NJ) was derived from pork intestine. Frog Ringer's solution contained (mM): NaCl (111), KCl (2.4), $MgCl_26H_2O$ (1.0), $CaCl_2$ $2H_2O$ (1.1), $NaHCO_3$ (0.195), glucose (5.5), and [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)]-Na-HEPES (5.0). NaCl, KCl, $MgCl_2$ and $NaHCO_3$ were obtained from Fisher (Fair Lawn, NJ), glucose from Sigma, and HEPES acid and Na-HEPES from Research Organics (Cleveland, OH).

Fluorescently labeled LDL was prepared fresh weekly as described by Pitas et al. (29). 1,1'-Dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine (Dil) was obtained from Molecular Probes (Eugene, OR). Dil binds to LDL by exchanging with phospholipid on the LDL particle. Dil does not transfer to other lipoproteins because the two octadecyl moieties make the compound extremely hydrophobic (30, 31). The spectral properties of Dil are similar to those of rhodamine (excitation maximum 540 nm and emission maximum 556 nm).

Dextran (20,000 mol wt) labeled with fluorescein isothiocyanate (FITC) was obtained from Sigma. The spectral properties of this compound are excitation maximum of 489 nm and emission maximum of 515 nm. Bovine serum albumin was labeled with tetramethylrhodamine isothiocyanate (32). Tetramethylrhodamine isothiocyanate (TRITC) was obtained from Research Organics.

Experimental preparation

Male leopard frogs (*Rana pipiens*) were prepared as described previously (33). In brief, the frogs were pithed and placed supine on a cork-lined tray that contained a clear quartz pillar. After the frog was secured in place, a lateral flank incision was made and the small intestine was floated out of the abdominal cavity with frog Ringer's solution. The small intestine was then draped over the quartz pillar for viewing by trans-illumination and epi-illumination. Thereafter, a continuous superfusion of frog Ringer's solution was applied to the small intestine. The frog and tray were transported to the experimental rig and the mesenteric microvessels were observed by trans-illumination. A "Y" branch of a venular microvessel was chosen for cannulation with micropipets using micromanipulators and hydraulic microdrives. One arm of the Y branch was chosen for cannulation with a micropipet containing a clear, nonfluorescent solution and called the washout micropipet. The other arm of the Y branch was cannulated with a micropipet containing a fluorescently

labeled macromolecule (e.g., LDL) and called the dye micropipet (**Fig. 1**).

After cannulation of the microvessel with both washout and dye micropipets, the light source of the trans-illumination pathway was turned off and a shutter was opened to the epi-illumination pathway. Thus, fluorescently labeled molecules perfused into the microvessel were excited by a mercury light in the epi-illumination pathway. The spectral properties of the excitation light were changed by inserting different filter cubes into the light pathway. The fluorescence image was transmitted via a beamsplitter to a Dage low-light television camera and a Nikon P101 photometer (40:60 split). The microvessel was positioned so that an adjustable photometric window was centered on a section of the microvessel downstream from the two arms of the Y branch (Fig. 1). Thus, fluorescence in a defined portion of the microvessel was simultaneously imaged with the camera and measured with the photometer.

Each microvessel was visually examined for leakage of dye from cannulation sites and surrounding microvessels. Fluorescent dye leaks were also detected by sudden changes in recording photometric fluorescence intensity delayed with respect to time of filling the microvessel lumen. If the problem of dye leakage could not be remedied by repositioning the micropipet, the experiment on that microvessel was abandoned. Only microvessels that were free of adherent or extravasated leukocytes were used in these studies.

Measurements of rates of vascular tissue LDL accumulation and efflux

Fluorescence intensity (I_f) in the photometric measuring window was determined over time (t) (**Fig. 2A**). I_{f0} is the Dil-LDL fluorescence intensity when Dil-LDL perfusion is initiated and Dil-LDL is assumed to be entirely within the vascular lumen. I_{fmax} is the maximal fluorescence intensity during perfusion with Dil-LDL. $I_{fmax} - I_{f0} = \Delta I_f$ is an estimate of the amount of LDL that is within or has crossed the endothelial layer. Therefore, the rate of LDL accumulation equals $\Delta I_f/t$ multiplied by the specific fluorescence for LDL (X). In the following equation N equals the number of vascular tissue LDL molecules.

$$N/t = (I_{fmax} - I_{f0})\,(X)$$

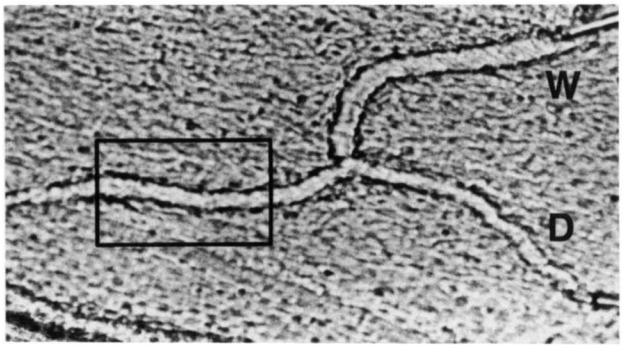

Fig. 1. Photomicrograph using trans-illuminated light of a converging venular capillary. One arm of the Y branch has been cannulated with a micropipet containing a clear nonfluorescent solution and termed the washout micropipet (W). The other arm of the Y branch has been cannulated with a micropipet containing a solution of Dil-LDL and termed the dye micropipet (D). The photometric measuring window is positioned along the straight portion of the microvessel. The diameter of the vessel in the window is approximately 15 μm.

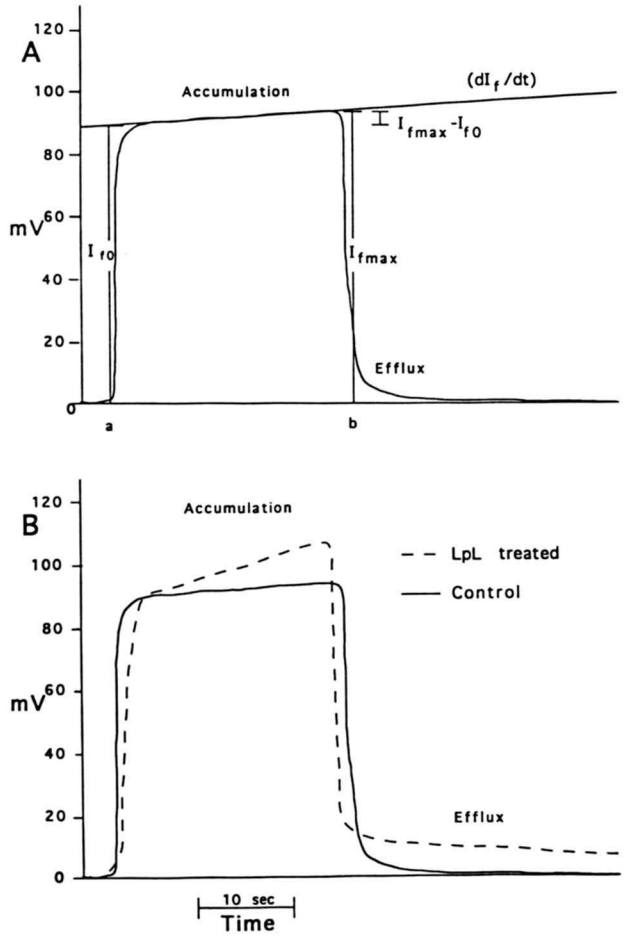

Fig. 2. A: Chart recording of the photometric output over time in the defined window positioned along the straight portion of the vessel in Fig. 1. Initially the microvessel is perfused with the clear, nonfluorescent solution and a baseline level of fluorescence intensity is determined. a. The microvessel is filled with Dil-LDL. I_{f0} is the fluorescence intensity immediately after the microvessel is filled with dye and represents the number of fluorescent molecules filling the lumen. b. Dil-LDL is washed out of the microvessel with the clear nonfluorescent solution and a step decrease in fluorescence intensity is seen. The maximal increase in fluorescence intensity (I_{fmax}) is measured immediately before washout with the nonfluorescent solution. $I_{fmax} - I_{f0}$ determines vascular tissue LDL accumulation during the Dil-LDL perfusion. LDL efflux from the vascular tissue is determined by the reduction of fluorescence intensity over time after washout of the vessel lumen with the nonfluorescent solution. B: A comparison of the chart recordings of the photometric output of the same segment of microvessel during a control Dil-LDL perfusion (same recording as in A) and during a Dil-LDL perfusion after the vessel had been treated with LpL (10^{-5} g/ml) for 9 min. The rate of LDL accumulation (N/t) is increased and LDL efflux is reduced after LpL treatment in this vessel when compared to control.

Therefore, in a given vessel where the LDL perfusion concentration remains constant, the rate of LDL accumulation (N/t) can be determined sequentially over time.

After Dil-LDL has been washed out of the vessel lumen with the clear buffer solution, a reduction in fluorescence intensity is seen which represents efflux of the Dil-LDL from the vessel wall (Fig. 2A). The rate of LDL efflux can be estimated by determination of the time interval during which there has been a reduction of 50% of Dil-LDL fluorescence intensity ($I_{fmax} - I_{f0}/2$). Thus, the greater the half-time of fluorescence decay ($T_{½}$), the slower the LDL efflux.

Protocols

Control measurements of the rate of LDL accumulation (N/t) and efflux ($T_{½}$) were made by cannulating individual microvessels with a washout micropipet containing frog Ringer's solution plus 10 mg/ml bovine serum albumin. The dye micropipet contained LDL labeled with Dil and was diluted with frog Ringer's solution. Bovine serum albumin was added to the dye solution to a final concentration of 10 mg/ml. Three to six measurements of N/t and $T_{½}$ were made for LDL perfusions of 30–45 sec at microvessel hydrostatic pressures of 10–15 cm H_2O. The effect of LpL on LDL accumulation and efflux was examined in four protocols.

1. Effect of LpL on LDL accumulation and efflux

After measurements of N/t and $T_{½}$ for control LDL perfusions were performed, the washout micropipet was exchanged for one containing LpL mixed with buffer solution. Repeat measurements of N/t and $T_{½}$ were obtained for subsequent LDL perfusions. Additional experiments were performed where the dye micropipet was exchanged for one containing LpL mixed with Dil-LDL and then the vessel was perfused with the mixture. All perfusions of the vessel were performed at the same hydrostatic pressure. Since N/t and $T_{½}$ were determined in each vessel at control and during LpL treatment, each vessel serves as its own control.

2. Pretreatment of vascular tissue with LpL

To examine the effect of LpL or LDL-LpL (see below) on vascular wall permeability, we determined macromolecular apparent permeability coefficients (P_s: cm/sec × 10^{-7}) before and after treatment with LpL or LDL-LpL. The procedures for determination of P_s have been described previously (33). Reference labeled macromolecules, which have been extensively characterized in our laboratory (3, 4, 34), were FITC-dextran (20,000 MW) and TRITC-albumin.

To determine the effect of LpL on vessel permeability in the absence of LDL, control measurements of P_s were made during FITC-dextran perfusions; the washout micropipet was removed and recannulated with a micropipet containing LpL diluted with frog Ringer's solution plus 10 mg/ml albumin. Multiple repeat measurements of P_s dextran were obtained after the vessel had been perfused with LpL.

3. Effect of LDL-LpL on albumin permeability

To determine whether LDL-LpL affected microvessel permeability to albumin, we determined TRITC-albumin apparent permeability before and after exposure of the microvessel to LDL-LpL. Initially, control albumin P_s was obtained after a microvessel was cannulated with a washout micropipet containing frog Ringer's solution plus 10 mg/ml unlabeled albumin and a dye micropipet that contained TRITC-albumin in frog Ringer's solution. Unlabeled albumin (4 mg) was added to TRITC-albumin to give a final concentration of labeled and unlabeled albumin of 10 mg/ml. After 3–6 measurements of albumin P_s were obtained, the washout micropipet was exchanged for one containing unlabeled LDL plus LpL and repeat measurements of albumin P_s were made.

4. Effects of heparin on LDL-LpL accumulation

Heparin prevents LpL interaction with heparin sulfate proteoglycans. Thus, adding heparin to the LDL-LpL mixture might affect LDL accumulation. Initially, control measurements of N/t were obtained with Dil-LDL in the dye micropipet. Next, the dye micropipet was removed and exchanged for one containing heparin, Dil-LDL, and LpL. Multiple measurements of N/t were again obtained. Then, the dye micropipet was removed and the vessel was cannulated with one containing Dil-LDL and LpL without heparin and N/t was measured.

RESULTS

LDL accumulation in LpL-treated vessels

The effect of LpL concentration on LDL accumulation (N/t) and efflux ($T_{½}$) was determined in three individually perfused blood vessels (**Fig. 3A and B**). N/t was determined in each vessel during Dil-LDL perfusions of 30–45 sec at control and after LpL was added to the buffer solution at concentrations of 10^{-9} g/ml, 10^{-7} g/ml, and 10^{-5} g/ml. N/t increased in two of three vessels after treatment with 10^{-7} g/ml LpL and increased in all three vessels after treatment of the vessel with 10^{-5} g/ml LpL.

LDL efflux was determined in these same three vessels for each LDL perfusion at the LpL concentrations noted above (Fig. 3B). Efflux was determined after the LDL solution has been washed out by the buffer solution and was quantitated as the time required for reduction of half of the Dil-LDL fluorescence intensity within the vessel wall ($T_{½}$; sec). $T_{½}$ was dependent on LpL concentration indicating reduced LDL efflux with higher LpL concentrations. Additionally, increased LDL accumulation was ac-

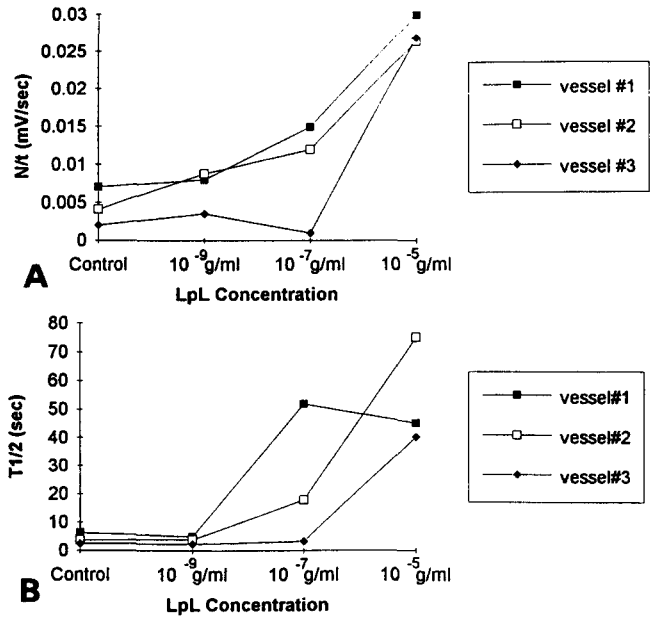

Fig. 3. Effect of LpL concentration on (A) LDL accumulation (N/t) and (B) LDL efflux ($T_{½}$) in three individually perfused vessels. In each vessel N/t and $T_{½}$ were determined for LDL perfusions at control and after LpL was added to the washout buffer solution in concentrations of 10^{-9} g/ml, 10^{-7} g/ml and 10^{-5} g/ml. Multiple LDL perfusions (3–10) were performed at control and at each LpL concentration. N/t and $T_{½}$ appeared to be dependent on LpL concentration.

companied by decreased LDL efflux. These studies suggested that LpL increased LDL binding to vascular tissue.

The effect on LDL accumulation was a function of LpL perfusion time. For example, N/t was obtained from a single vessel during control LDL perfusions and after treatment with LpL where sequential perfusions of LDL were performed from 2–30 min after initiation of LpL treatment. N/t for three LDL perfusions at control was 0.004 ± 0.001 (SD) mV/sec. Then, the washout micropipet was exchanged for one containing LpL (10^{-5} g/ml) in buffer solution and repeat measurements of N/t were made at 2, 3, 9, 15, 19, and 30 min. N/t during LpL treatment was 0.01, 0.007, 0.029, 0.051, 0.076, and 0.043 mV/sec, respectively. Fig. 2B demonstrates LDL accumulation in the same vessel during a control perfusion and a perfusion performed 9 min after the washout micropipet had been exchanged for one that contained LpL. A linear rate of increase in fluorescence intensity was seen during the control LDL perfusion and for the LDL perfusion after LpL treatment. N/t increased 0.05 mV/sec in the control LDL perfusion and 0.29 mV/sec when measured 9 min after perfusion with LpL. As demonstrated in this example, treatment of vessels with LpL for greater than 9 min resulted in dramatic increases in rate of LDL accumulation. These data suggest that time was required for LpL interaction with molecules within vascular tissue. We postulate that LpL within the vessel lumen must cross the endothelial cell barrier and associate with subendothelial proteoglycans. Then, the proteoglycan-LpL complex binds LDL after it crosses the endothelial barrier thereby creating LDL retention sites.

Similar results were seen when LpL was mixed with LDL prior to perfusion of the vessel. Perfusion of seven separate blood vessels with the mixture of LDL-LpL increased the rate of LDL accumulation (N/t) in every blood vessel tested when compared to control LDL perfusions (**Table 1**). The ratio of N/t after LDL-LpL perfusions to control LDL perfusions ranged from 3 to 50.5 (mean 15.2). For the entire group of seven vessels, N/t increased from 0.024 ± 0.005 (SD) mV/sec to 0.34 ± 0.31 (SD) mV/sec when LpL was added to Dil-LDL. In every experiment the addition of LpL increased Dil-LDL N/t, however, the amount of increase was variable. This variation in effect could be related to localized differences in vessel constituent elements such as amount of proteoglycans or vessel permeability. In general, serial measurements of N/t increased in value during the initial 9 min of perfusion with the mixture of LDL-LpL. After 9 min, N/t appeared to plateau.

Effects of LpL and LpL-LDL solutions on dextran and albumin flux

To determine whether LpL alone had effects on macromolecular flux, the apparent permeability coefficients (P_s) of FITC-dextran (20,000 MW) were compared before and after exposure to LpL (n = 2 microvessels). No significant difference in P_s dextran before and after exposure to LpL (2.0 ± 2.0 (SD) × 10^{-7} cm/sec vs. 3.4 ± 2.0 (SD) × 10^{-7} cm/sec, respectively) were seen. These data demonstrate that LpL alone appeared to have no direct effects on the vascular wall permeability.

To determine the effect of LDL-LpL on vascular wall permeability, P_s albumin values were determined before

TABLE 1. Rate of accumulation of LDL in blood vessels

	N/t		
Microvessel	LDL	LDL-LpL	Ratio:LDL-LpL/LDL
	mV/sec		
1	0.02	0.14	7.0
2	0.02	1.01	50.5
3	0.03	0.48	16.0
4	0.02	0.06	3.0
5	0.02	0.43	21.5
6	0.03	0.11	3.7
7	0.03	0.15	5.0

The rate of accumulation of Dil-LDL (N/t) was determined in seven microvessels from seven different animals for control Dil-LDL (protein = 0.72 mg/ml) perfusions and for perfusions after the dye micropipet was exchanged for one containing Dil-LDL (same concentration) + LpL (10^{-5} g/ml). The ratio of N/t after LpL-LDL perfusions to control LDL perfusions (LDL-LpL/LDL) is presented in the fourth column. The increase in LDL accumulation after addition of LpL was significant ($P < 0.01$) when analyzed using a paired t test.

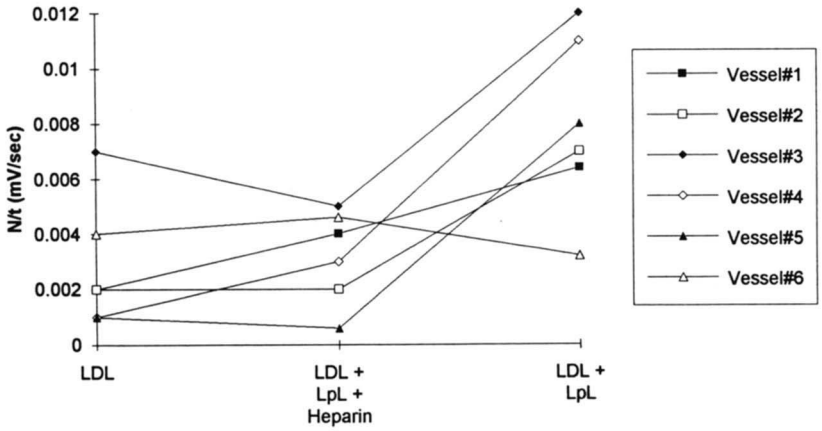

Fig. 5. Effect of heparin on LpL-mediated LDL accumulation. To determine whether heparin (an inhibitor of LpL binding to proteoglycans) alters accumulation of LDL, we compared LpL-mediated LDL accumulation in the presence and absence of heparin. N/t was determined in six vessels during LDL (1.05 mg/ml protein) perfusions, LDL + LpL (10^{-5} g/ml) + heparin (10 units) perfusions (denoted LDL + LpL + heparin) and LDL + LpL perfusions (same concentrations) without heparin. As is apparent, LpL did not significantly increase LDL accumulation in the presence of heparin.

and after exposure of the microvessel to unlabeled LDL and unlabeled LpL. In three individual microvessels, P_s albumin did not change significantly before (3.8 ± 0.7 (SD) $\times 10^{-7}$ cm/sec) or after (3.2 ± 1.0 (SD) $\times 10^{-7}$ cm/sec) exposure to LDL-LpL. Thus, the effect of LpL on LDL accumulation does not appear to be mediated by increased microvessel permeability.

Imaging fluorescent LDL in the vessel wall

We further explored whether the marked changes in vascular tissue LDL accumulation were due to reduced LDL efflux by imaging perfused vessels. As shown in Fig. 2B, I_f (fluorescence intensity) at the beginning of the washout period was greater for the LpL-treated vessels. In addition, the rate of decline in fluorescence intensity (denoted Efflux on Fig. 2B) was dramatically and constantly reduced in LpL-treated vessels. To quantify this effect, the fluorescence intensity over time was plotted in the same vessel and the curves were fitted using a logarithmic function for a control LDL perfusion ($e^{-0.231}$) and LDL perfusions 9 min after LpL treatment ($e^{-0.077}$). During this experiment the control half-life of the Dil-LDL decay was 5 sec. The half-life of Dil-LDL decay in the LpL-treated vessels was 24 sec after 6 min of LpL perfusion and $>$ 60 sec after 9 min of LpL perfusion. Therefore, LpL markedly decreased LDL efflux and this appears to be the principal process responsible for the increased LDL accumulation.

LpL-mediated increases in LDL retention were imaged by a low-light television camera during the experiment above and were saved on videotape. Shown in **Fig. 4** are images of the same vessel at control and after 9 min LpL treatment. The control panel shows the vessel during perfusion with Dil-LDL (30-sec perfusion) and at 10 sec after the Dil-LDL was washed out of the lumen of the vessel. No vascular tissue Dil-LDL is seen at 10 sec after washout. The LpL-treated panel shows the vessel under the same optical conditions during perfusion with Dil-LDL (30-sec perfusion) and at 10 sec, 30 sec, 1 min, and

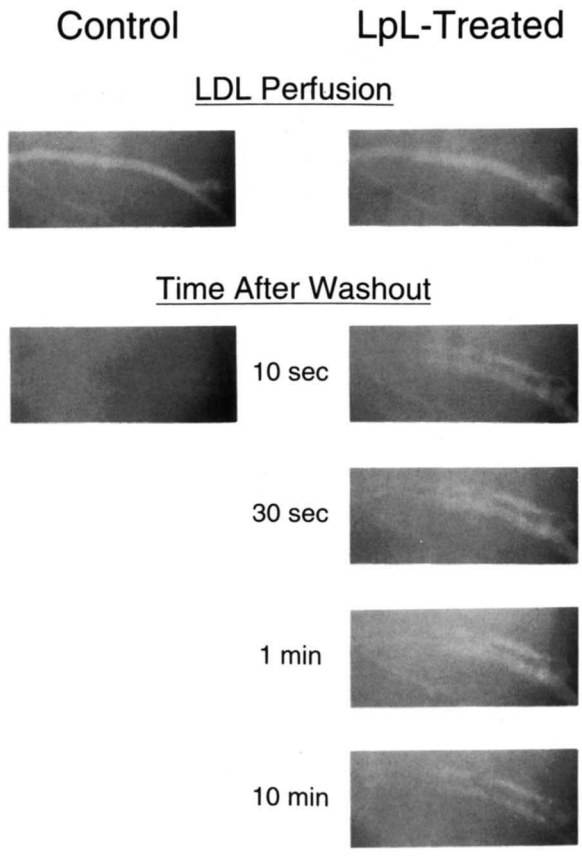

Fig. 4. Real time images of a segment of microvessel before and after LpL treatment. Dil-LDL was excited with an Hg light and fluorescence was filtered through a rhodamine filter cube. Images were captured with a low-light television camera where automatic control circuits (gain, sensitivity, and black level) were disabled and all three were kept under manual control and not changed during the course of the experiment. The video images were stored on videotape for later off-line analysis. The control panel shows the vessel during Dil-LDL perfusion and at 10 sec after washout of the vessel lumen with the nonfluorescent solution. The LpL-treated panel shows the same vessel during perfusion with LpL (10^{-5} g/ml) + Dil-LDL and at 10 sec, 30 sec, 1 min, and 10 min after washout of the Dil-LDL.

10 min after washout of the Dil-LDL from the vessel lumen. Increased fluorescence due to LDL in the vascular tissue is noted 10 sec after washout and remained for greater than 10 min in the LpL-treated vessel. Thus, either the interaction of LpL with LDL prior to or during the perfusion increased LDL accumulation by preventing the efflux of LDL from vascular tissue.

Effect of heparin on LDL movement

To test whether LDL accumulation in the presence of LpL required LpL binding to subendothelial proteoglycans, control perfusions (Dil-LDL alone), perfusions with solutions of Dil-LDL + LpL + heparin (denoted LDL + LpL + heparin in **Fig. 5**), and perfusions with solutions of Dil-LDL and LpL without heparin were sequentially performed. Three to seven measurements of N/t were made with each treatment. In our other experiments perfusion with LpL-LDL solutions led to a consistent increase in LDL accumulation; however, this effect was reduced or eliminated when heparin was present in the six individually perfused vessels. N/t was not significantly different for control LDL perfusions and for LDL perfusions after mixture of LpL and heparin when analyzed by ANOVA with repeated measures. However, treatment of the vessels with LpL and LDL resulted in increased N/t that was significantly different from N/t for LDL perfusions and LDL + LpL + heparin perfusions ($P = 0.004$). It should also be noted that compared to vessels that had not been exposed to heparin-containing solutions, perfusions with LpL-LDL led to less marked increases in LDL accumulation (compare Fig. 5 with Table 1). This might have resulted from residual heparin remaining in the vascular tissue when the micropipets were exchanged.

DISCUSSION

In our studies perfusion of LpL or a mixture of LDL-LpL increased LDL accumulation in vascular tissue. This increased accumulation was apparent during the time when the vessels were perfused with fluorescent LDL and could be directly visualized in the images of vessels obtained after Dil-LDL was cleared from the lumen of the vessel by perfusing the vessel with the nonfluorescent solution. It should be noted that rather than forming an intense outline of the vessel, the retained Dil-LDL was found in a diffuse pattern which extended outside the lumen of the vessel. Thus, rather than causing LDL to bind to the luminal aspect of the vascular wall, LpL led to LDL retention in subendothelial areas. This is likely the result of transfer of both LpL and LDL to the subendothelial space.

Changes in the permeability of endothelial cells to macromolecules including LDL and albumin have been investigated using both cultured endothelial cell monolayers and in vivo perfused vessels. However, to the best of our knowledge, there are no reports of interventions which specifically alter the retention, or efflux, of molecules within vascular tissue. Our method for quantitating LDL efflux was to first perfuse the vessel with Dil-LDL, then wash out the lumen of the blood vessel with a nonfluorescent solution. All Dil-LDL left in the photometric window after the washout was therefore within vascular tissue or bound to the luminal side of the endothelium. However, fluorescence microscopy confirmed that most fluorescently labeled LDL was in the subendothelial space. The loss of fluorescence intensity over time was defined as LDL efflux from vascular tissue. Our short-term studies indicated that both control and LpL-treated microvessels exhibited exponential decay of Dil fluorescence during the period of LDL efflux. This is expected in our system as the Dil-LDL that had accumulated in the vascular tissue was a nonrenewable resource and Dil-LDL efflux would be expected to have an exponential fluorescence decay. The rate of decay of Dil-LDL in LpL-treated vessels was reduced relative to control. In fact, in some studies, despite a washout period of several minutes, LpL caused persistent retention of Dil-LDL. Therefore, much of the accumulation of Dil-LDL within vascular tissue seen with perfusions containing LpL was due to reduced efflux of Dil-LDL.

The best known function of LpL is to hydrolyze triglyceride and phospholipids in circulating lipoproteins leading to production of free fatty acids, di- and monoglycerides, and lysophospholipids. However, we tested the possibility that LpL-mediated hydrolysis of LDL-generated lipolysis products altered vascular tissue permeability. There was no change in dextran and albumin permeability in vessels that were perfused with LpL and LpL-LDL solutions. This strongly suggests that LDL accumulation was not due to permeability changes in the endothelial barrier. In other experiments, LpL-LDL solutions were perfused into vessels in the presence of heparin. At the concentrations used, heparin reduces the interaction of LpL with heparan sulfate proteoglycans but does not alter LpL enzymatic actions. Because heparin markedly reduced Dil-LDL accumulation within vascular tissue, our data are most consistent with the hypothesis that LpL anchors LDL to subendothelial matrix via molecular interactions that are exclusive of its enzymatic functions.

Our studies were performed in a heterologous perfusion system and, as such, were not physiologic nor were they a model for atherosclerosis. Our goal was to examine basic biophysical processes associated with flux of LDL through the vascular wall. These studies were the first in vivo, in situ experiments to examine LDL-LpL interaction with individually perfused blood vessels. Because of the nature of the experiments, multiple measurements of LDL accumulation were made at control and after ex-

posure of the vessel to LpL. Thus, paired comparisons of LDL accumulation were performed in each vessel which greatly simplified the analyses. The composition of the perfusion and superfusion solutions were exactly known and microvessel hydrostatic pressure was kept constant as higher hydrostatic pressures increase LDL influx across the microvascular barrier (34). Our in vivo experiments have allowed us to control these important variables, all of which cannot be accounted for in cell culture, whole organ, or ex vivo experiments.

The experimental preparation and analysis presented in this paper provides an in vivo, in situ test system to examine lipoprotein-vascular wall interactions. This system should have value in the future for examining conditions that modulate the lipoprotein and/or the vascular wall. In order to make these studies more relevant to human physiology and metabolism, additional studies are needed in a mammalian large artery perfusion model using homologous lipoproteins.

Because LpL activity, derived from foam cell macrophages, is increased in atherosclerotic lesions, Zilversmit (14) postulated that LpL promotes atherosclerosis. He hypothesized that local hydrolysis of chylomicrons generates atherogenic remnant lipoproteins which then promote the accumulation of lipid with the vascular wall. In contrast, studies in tissue culture (6, 26) demonstrated that LpL increased the association of LDL and very low density lipoproteins with subendothelial cell matrix. LDL retention in the matrix decreased when heparin or anti-LpL antibodies were included in the culture medium. These findings suggest that LpL formed a molecular bridge with the lipid-binding region of LpL attached to LDL and the heparin-binding LpL domain attached to heparan sulfate proteoglycans within the matrix. Our current studies clearly show that LpL also increases LDL retention in vascular tissue. Thus, our data support the hypothesis that vascular wall LpL promotes atherogenesis.

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