Immunocytochemical localization of the functional fraction of lipoprotein lipase in the perfused heart[1,2]

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Abstract The functional (heparin-releasable) fraction of myocardial lipoprotein lipase (LPL) has been located at the lumen surface of capillary endothelium by means of an indirect immunocytochemical perfusion method for electron microscopy. The primary step immunoreactant was an IgG fraction of goat antiserum directed against LPL from rat heart. The second step antibody, conjugated with horseradish peroxidase, was rabbit IgG directed against goat IgG. Peroxidase reaction product, when present, appeared at the surface and in invaginations of the lumenal plasma membrane of capillary endothelium and also on chylomicrons adherent to that membrane. The highest coverage by such product occurred when the highest heparin-releasable heart LPL activity was attained after fat-feeding of rats. Coverage was low when a low level of heparin-releasable heart LPL activity was induced by carbohydrate-feeding. Coverage was very low in the perfused hearts after heparin-release of functional LPL activity. The positive association between these immunocytochemical results and actual levels of functional LPL activities indicates that functional LPL in the isolated rat heart is at the lumen surface of capillary endothelium.—**Pedersen, M. E., M. Cohen, and M. C. Schotz.** Immunocytochemical localization of the functional fraction of lipoprotein lipase in the perfused heart. *J. Lipid Res.* 1983. **24:** 512–521.

Supplementary key words immunocytochemistry • capillaries • endothelium • chylomicrons

Lipoprotein lipase (LPL) is generally considered to be present at the lumen surface of capillary endothelium and to be involved in hydrolysis of adherent chylomicron and very low density lipoprotein triglycerides. Evidence for this location as the functional site of LPL includes the rapidity with which passage of heparin through capillary beds releases LPL activity in vivo or in vitro (1) and the subsequent loss of the capacity to hydrolyze chylomicron triglycerides (2). Additional support for the capillary lumen surface location for functional LPL is derived from the ability of injected LPL antiserum to block removal from plasma of very low density lipoprotein triglyceride (3) and the ability of LPL antibodies, perfused through the heart, to block hydrolysis of subsequently perfused chylomicron triglyceride (4).

No immunocytochemical visualization of LPL at the submicroscopic level has been reported. Histochemical visualization of inferred LPL activity associated with capillary beds has been based on the Gomori (5) method of metal salt precipitation of released free fatty acids. Under optimum conditions for LPL activity, this histochemical reaction in mouse adipose tissue was negative in fat cells but positive in capillaries (6); the inference is that functional LPL was present in these capillaries. In the same study the biological control material from fasted mice showed "relative absence of capillary activity" but a heavy reaction of fat cells, indicative of the lipase activity of triglyceride mobilization. Positive histochemical reactions involving capillaries have also been obtained by electron microscopy with lactating mouse mammary gland after intravenous injection of Intralipid (7) and with chylomicrons perfused through rat parametrial adipose tissue (8). Although this histochemical approach may reveal free fatty acids in tissues, such an approach would fail to demonstrate LPL-active sites lacking adherent chylomicrons or very low density lipoproteins.

An immunohistochemical demonstration of an endothelial enzyme with "lipase-like" activity has been reported (9). When an antiserum against this enzyme, a pig adipose tissue lipase, was used as primary step antiserum in light microscopic indirect immunohistochemistry of various pig tissues, a positive reaction was

Abbreviations: IgG, immunoglobulin G; KRB, Krebs-Ringer bicarbonate buffer; LPL, lipoprotein lipase; SDS, sodium dodecyl sulfate.

[1] A portion of this work was presented at the meeting of the American Society of Biological Chemists in New Orleans, June 1980, and appears in abstract form in *Federation Proc.* **39:** 1726.

[2] Part of the data in this study is from a thesis submitted by M.E.P. in partial fulfillment of the requirements for the Ph.D. degree in the School of Public Health, University of California, Los Angeles.

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evident in endothelium of myocardial capillaries, aorta, and intercostal arteries, and at the surfaces of adipocytes and skeletal muscle cells but not in liver, lymph nodes, spleen, or kidney. The antiserum blocked all of the pH-6.5 activity of the purified adipose tissue enzyme in hydrolysis of tributyrin and 70% of that activity of a similarly prepared myocardial lipase. Some properties of the adipose tissue lipase were similar to those of LPL. Whether these enzymes are releasable by heparin has not been reported.

We describe here the indirect immuno-electron microscopic localization of functional LPL at capillary lumen surfaces in isolated rat hearts and the positive association between extents of immunocytochemical reaction and levels of heparin-releasable LPL[5] resulting from fat- (10) or glucose-feeding (11) or heparin perfusion.

MATERIALS AND METHODS

Heart perfusion

Male Sprague-Dawley rats, 150–250 g, (Hilltop Lab Animals), were kept on a 12-hr light (0630–1830) dark (1830–0630) cycle. The animals, maintained on Purina rat chow ad libitum, were fasted 16–18 hr and then intubated with 3 ml of 60% (w/v) glucose or 2 ml of safflower oil. Immediately after the fasting period or 3 hr after intubation, hearts were removed and then retrograde aortal perfusion begun (2, 11). For the biochemical studies, initial perfusion was with KRB, 8 ml/min, for 2 min at 37°C to wash out blood and establish a stable heartbeat. Then heparin (Calbiochem-Behring Corp., San Diego, CA), 1 unit per ml KRB, was perfused through the heart for 30 sec at the same rate to obtain the perfusate for assay of heparin-releasable LPL activity. For the studies in which heparin-releasable LPL activity was removed prior to the immunocytochemical procedure, hearts isolated from fasted rats were perfused 2 min with the heparin solution described above. In other similarly perfused hearts, heparin-releasable activity was assessed in a subsequent 30-sec heparin perfusate.

LPL assay

The assay (12) employed serum-activated triolein containing a trace amount of tri[9,10(n)-^{3}H]-oleoylglycerol (446 mCi/mmol, Amersham, Chicago, IL). One mU of LPL activity represents the release at 37°C of 1 nmol free fatty acid per min.

[5] In this study the terms functional LPL and heparin-releasable LPL are used interchangeably.

Purification of heparin-releasable LPL

Perfusates from the first 40 sec of heparin perfusions of hearts isolated from 25 rats fed safflower oil were pooled. The pool was partially purified by affinity chromatography on heparin-Sepharose 4B (13). The LPL activity was eluted with 5 mM sodium barbital, 20% (w/v) glycerol, pH 7.2, designated "buffer 1" containing 1.2 M NaCl. The peak fractions of LPL activity were combined and then diluted with buffer 1 to a final concentration of 0.75 M NaCl.

Purification and characterization of anti-LPL antibody

The IgG fraction of a goat anti-rat heart LPL antiserum was isolated (4). Analysis of this fraction by immunodiffusion in agarose gel (14) revealed the presence of antibody directed against a component of rat serum. In order to remove anti-rat serum antibodies, the IgG fraction was passed through a column of Sepharose 4B to which rat serum had been covalently linked (15). Immunodiffusion of the eluate showed no remaining precipitation line against rat serum. Although this eluate still retained the capacity of the original IgG to inhibit rat heart LPL activity, it produced no immunodiffusion precipitation line with LPL purified (4) from rat heart tissue. This purified anti-LPL IgG was used for the biochemical studies and also as primary step immunoreactant in the indirect immunocytochemical studies. Serum IgG obtained from the same goat prior to injections of LPL was isolated and purified by the same procedure used to prepare anti-LPL IgG. This preimmune IgG was employed as a control for primary step immunoreactant in the immunocytochemical experiments. Protein was estimated by the method of Bradford (16).

Because the Ouchterlony double diffusion between anti-LPL IgG and rat heart LPL was negative, an attempt was made to characterize the anti-LPL IgG by its affinity for protein components of partially purified heparin-releasable LPL. Anti-LPL IgG, 30 mg, was covalently linked to 35 ml of Sepharose 4B (15). Partially purified LPL (3600 mU) in buffer 1 containing 1.2 M NaCl was diluted to a final concentration of 0.75 M NaCl, then added to 6 ml of the antibody-linked gel suspension. The mixture was gently swirled at 4°C for 2 hr and then washed on a sintered glass funnel with buffer 1, 30 ml containing 1 M NaCl, then 30 ml containing 0.15 M NaCl. The washed gel, dispersed in the latter solution, was poured into a column and allowed to settle. A 0.2 M glycine-HCl buffer, pH 2.8, containing 0.5 M NaCl, was passed through the column, and 10-drop fractions were collected directly into 57 μl 1.39 M Tris-HCl buffer, pH 8.4 (cf. 17). These fractions were

subjected to SDS polyacrylamide slab gel electrophoresis (18), followed by silver staining (19).

Immunocytochemical and fixation procedures

Perfusions for immunocytochemistry employed one-way gravity flow at 37°C unless noted otherwise.

Isolated hearts were perfused for 3 min, at 10 ml/min, with KRB containing 10 mM glucose, 0.2% (w/v) NaCl, and 4% (w/v) bovine serum albumin (fraction V powder, Sigma Chemical Co., St. Louis, MO); the total solution is termed "buffer 2". If the red color of the pulsating heart did not disappear quickly and uniformly, the heart was discarded. Fifteen ml of buffer 2 containing 8–15 mg of primary step anti-LPL IgG (or primary step preimmune IgG as a technical control), with or without 5 mg of normal rabbit IgG (lyophilized, Miles Laboratories, Research Products, Elkhart, IN), with adjustment of the albumin to bring total protein to approximately 600 mg, were recirculated at room temperature through each heart for 10 min at 4 ml/min. Next, hearts were perfusion-rinsed with buffer 2 for 4 min at 8 ml/min. This was followed by recirculating perfusion at room temperature for 10 min, at 4 ml/min, with 15 ml of buffer 2 containing second step horseradish peroxidase conjugate of rabbit IgG (0.075 or 0.20 mg) specific for heavy and light chain components of goat IgG (Litton Bionetics, Kensington, MD), with or without 5 mg of normal rabbit IgG, with adjustment of albumin as above. Hearts were then perfusion-rinsed for 4 min at 8 ml/min with 32 ml of KRB containing 2.6% (w/v) polyvinylpyrrolidone (PVP 40, pharmaceutical grade, Sigma Chemical Co., St. Louis, MO), 10 mM glucose, and 0.2% (w/v) NaCl.

From this point on, one-way perfusions were carried out with a Sorvall Peristaltic Pump (DuPont Instruments-Sorvall Biomedical Division, Newtown, CT) using ice-cold solutions, at rates of 4–6 ml/min, unless otherwise noted. Hearts were perfused as follows: 2% (w/v) glutaraldehyde (25% w/v EM grade glutaraldehyde, Scientific Chemical Co., Huntington Beach, CA) in modified Tyrode solution (20, cf. 21), pH 7.4, for 5 min; modified Tyrode solution, pH 7.4 for 3 min; 300 mM glycine at pH 10 (22) for 3 min; modified Tyrode solution for 3 min; for the peroxidase reaction (23), 0.03% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.01% (w/v) H_2O_2 in 0.05 M Tris buffer, pH 7.5, filtered just before use with a 500-ml Nalgene filter unit, 0.2 μm pore size (Nalgene Labware Division, Nalge Company, Rochester, NY), for 30 min; modified Tyrode solution, for 10 min. Fixation was resumed by one of two procedures; all solutions were ice-cold except where noted.

Procedure 1. Hearts were cut in a saggital direction and then immersed in glutaraldehyde-Tyrode solution. Thirty minutes later those mid-zones of the outer walls of the left ventricles which had become brown from the peroxidase reaction were cut into approximately 1-mm cubes. The blocks were immersed for at least 30 min in glutaraldehyde-Tyrode solution, followed by two 7-min rinses in modified Tyrode solution, 60 min in OsO_4 fixative (one part 4% (w/v) OsO_4 plus three parts pH 7.4 modified Tyrode solution), and, at room temperature, two 7-min rinses in modified Tyrode solution and then 0.2% (w/v) aqueous uranyl acetate for 60 min.

Procedure 2. Hearts were perfused, first with OsO_4 fixative, 3 ml/min for 10 min, then with modified Tyrode solution at the same rate for 3 min. Those mid-zones of the left and right ventricular walls which had been blackened by the OsO_4 perfusion were cut, under modified Tyrode solution, into approximately 1-mm cubes. The blocks were kept in OsO_4 fixative in vials, 1 to 2 hr, and then taken through the following steps at room temperature: two 7-min rinses with Michaelis buffer without NaCl, pH 7.3 (24); 0.5% (w/v) uranyl acetate in the same buffer (25), pH 5.2, for 60 min; two 7-min rinses in the same buffer.

Procedure 2 offered two advantages over Procedure 1: cell plasma membranes were much more visible, and the irregular scattering of small electron-dense particles in subendothelial tissues in most regions within blocks fixed by Procedure 1 was avoided.

After either fixation procedure, the blocks were processed for flat mold embedment in Epon 812 (26). The embedments were polymerized at 37°C for 12–20 hr and then at 60°C for at least 2 days.

Sections were cut at settings of 40 or 60 nm on a Sorvall Porter-Blum MT 2B Ultramicrotome (DuPont Instruments-Sorvall Biomedical Division, Newtown, CT), then mounted on 100-mesh copper slit grids bearing collodion substrates. Some sections were stained with alkaline lead citrate (27) and then all were coated with evaporated carbon. Sections were examined in Hitachi HS-8-2 and HU-11A Electron Microscopes (Hitachi Scientific Instruments, Nissei Sangyo America, Ltd., Mountain View, CA).

Estimation of immunocytochemical reaction

The extent of immunocytochemical reaction in capillaries was estimated for three groups of animals; namely, fat-fed (with an additional rat in this group used for a preimmune IgG technical control), glucose-fed, and fasted. The fasted group was used for heparin-perfusion of the isolated hearts prior to immunocytochemistry. Sampling for estimation of the immunocytochemical reaction was as follows: blocks of embedded tissue from each heart were picked in random order for sectioning at a thickness setting of 40 nm. Single sections

from the blocks were examined until three blocks were found that provided sections with numerous capillaries in approximate cross-sectional view. One unstained section from each of these three blocks per heart was examined systematically, starting at one corner of the section, then continuing back and forth along successive slits of the grid, until about one-third of the sample total of 100 capillaries per heart was evaluated. Only capillaries that were circular to slightly elliptical, as estimated by eye with the electron microscope and by comparison with a drawn ellipse of 1.5 axial ratio, were included in the sample. This reduced the chance that any capillary would be viewed more than once. Assessments of coverage of capillary lumen surfaces by immunoreaction product were made by one person (M.E.P.) by direct view in the electron microscope. Each capillary was assigned to one of five equal intervals between 0 and 100% coverage.

The data were arranged into frequency distributions. These distributions were analyzed in two different ways to see whether the extent of immunoreaction for LPL was related to activity of heparin-releasable LPL in other experimentally comparable hearts. In one analysis, median % coverage by immunoreaction product and a confidence interval covering the population median were estimated for each frequency distribution as described in the Appendix.[6] For the other analysis, data in the frequency distributions were arranged into contingency tables to test for independence vs. association between extent of immunoreaction for LPL and LPL activity.

RESULTS

Immunocytochemical localization of functional LPL

For the fat-fed rat, sites of functional LPL antigen in the isolated heart were presumably revealed by slight to heavy deposits of electron-dense peroxidase reaction product along the lumen surface of capillary endothelium and some of its lumenal invaginations, as well as within some vesicular profiles near that surface (**Fig. 1** and **Fig. 2**). In contrast, for a heart isolated from a glucose-fed rat, the immunocytochemical reaction was low to absent among capillary profiles (**Fig. 3**). With preimmune IgG substituted for primary step anti-LPL IgG, peroxidase reaction product was extremely low to absent in capillaries of hearts from either glucose- or fat-fed rats (**Fig. 4**). This control assessed the level of nonspecific reaction in such hearts. Also in the heart from which functional LPL had been removed by perfusion with heparin, the immunocytochemical reaction product was generally very low to absent among capillary profiles (**Fig. 5**).

Apparently, free chylomicrons were washed out of the heart capillaries during the initial perfusions with KRB. All chylomicrons remaining were adjacent or close to lumen surfaces of capillaries. Peroxidase reaction product was present at the peripheries of these chylomicrons (Figs. 1 and 2).

Estimates of capillary lumen surface coverage by immunocytochemical reaction product

As compared to the heparin-preperfused condition (**Table 1**), the observed frequency distributions show a shift toward higher extents of product coverage of capillary lumen surfaces corresponding to increases in heparin-releasable LPL activity. The shift is noticeable with glucose-feeding and particularly striking with fat-feeding. Half of the capillaries counted in a heart from a fat-fed rat were in the 60 to 100% coverage range, whereas no capillaries sampled from a glucose-fed rat were in that range. In a heart isolated from a fasted rat, then perfused with heparin prior to immunocytochemistry, 33 of 100 capillary profiles showed no reaction product. For the control heart, isolated from a fat-fed rat and then perfused with preimmune IgG instead of primary step anti-LPL IgG, 9 out of a sample of 100 capillary profiles were free of immunoreaction product and the others contained only 1 to 10 product particles per profile. This background level of coverage was less than 0.8%, assuming a spherical product particle diameter of 0.01 μm and 10 product particles on a circular capillary lumen surface profile of 4 μm diameter.

Because heparin preperfusion and glucose feeding produced skewed frequency distributions (Table 1), it appeared that the median would be the most appropriate single central measure for detecting a possible association between extents of immunocytochemical reaction and levels of functional LPL activities. Median % coverage was estimated for each of the observed frequency distributions in Table 1 as follows. A curve was fitted to each observed frequency distribution; Table 1 shows expected frequencies calculated from these fitted curves for comparison with observed frequencies. The equation for calculation of areas under each fitted curve was used to estimate the sample median % coverage as well as the confidence interval covering the corresponding population median % coverage. Details are given in the Appendix.[6]

Fig. 6 shows plots of the estimated sample median % coverage values, together with estimated 99.9% confidence intervals covering corresponding population

[6] See NAPS document no. 04086 for 15 pages of supplementary material (Appendix). Order from NAPS % Microfiche Publications, P.O. Box 3513, Grand Central Station, New York, NY 10163. Remit in advance, in U.S. funds only, $7.75 for photocopies or $4.00 for microfiche. Outside the U.S. and Canada, add postage of $4.50 for photocopy and $1.50 for microfiche.

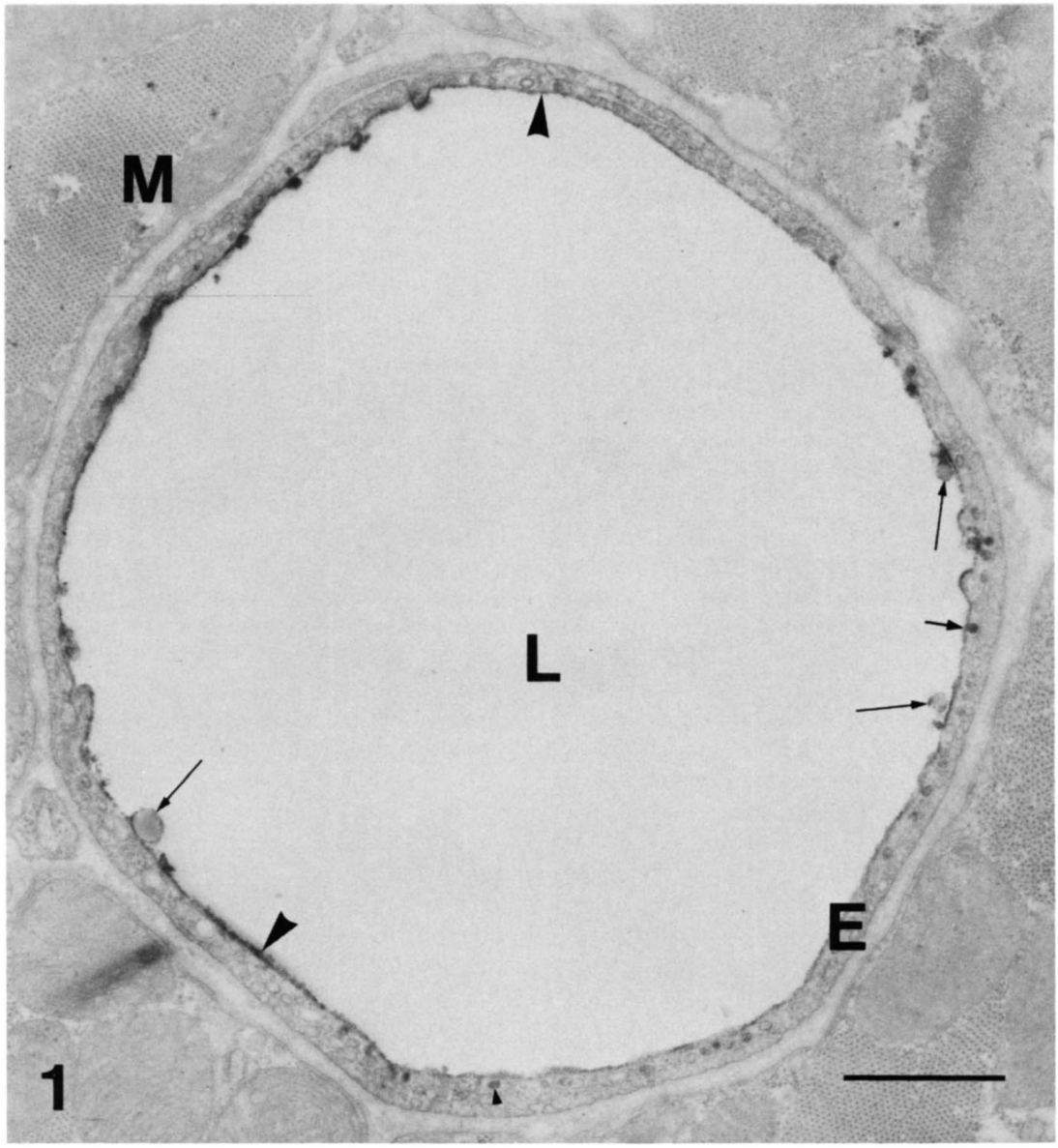

Fig. 1. Low magnification electron micrograph of a cross-section through a heart capillary from a fat-fed rat, showing indirect immunocytochemical localization of functional LPL at the lumen surface of the endothelium (large arrowheads). Electron-dense reaction product of the peroxidase-labeled second step antibody overlies nearby antigenic sites of LPL. Reaction product is also present in some lumenal plasma membrane invaginations (short arrow) and some cytoplasmic vesicular profiles (small arrowhead). The amount of immunocytochemical product varies along the lumen surface of the capillary. A few chylomicron-like particles with associated immunocytochemical product (long arrows) can be seen along that surface. Fixation procedure 2 (see Methods); section thickness 60 nm; section stained 3 min with alkaline lead citrate. Bar represents 1 μm. Capillary endothelium (E), capillary lumen (L), muscle cell (M).

median % values, vs. levels of heparin-releasable heart LPL activities. The confidence intervals of the preperfused, glucose-, and fat-fed groups do not overlap; taken together with the trend of the plotted points, this suggests a positive association exists between median % coverage and heparin-releasable LPL activity. The closeness of the plotted points to the straight regression line may be fortuitous since each of the upper two plotted points represents a functional LPL activity measurement on a single heart, whereas the lowest point represents a mean of functional LPL activity measurements on seven hearts.

This positive association between median % coverage and heparin-releasable LPL activity was substantiated by a different statistical analysis of the data in Table 1. Observed frequencies in this table were the basis for two 2 by 2 contingency tables (**Table 2**) for Model II G-tests for independence (28). For both contingency

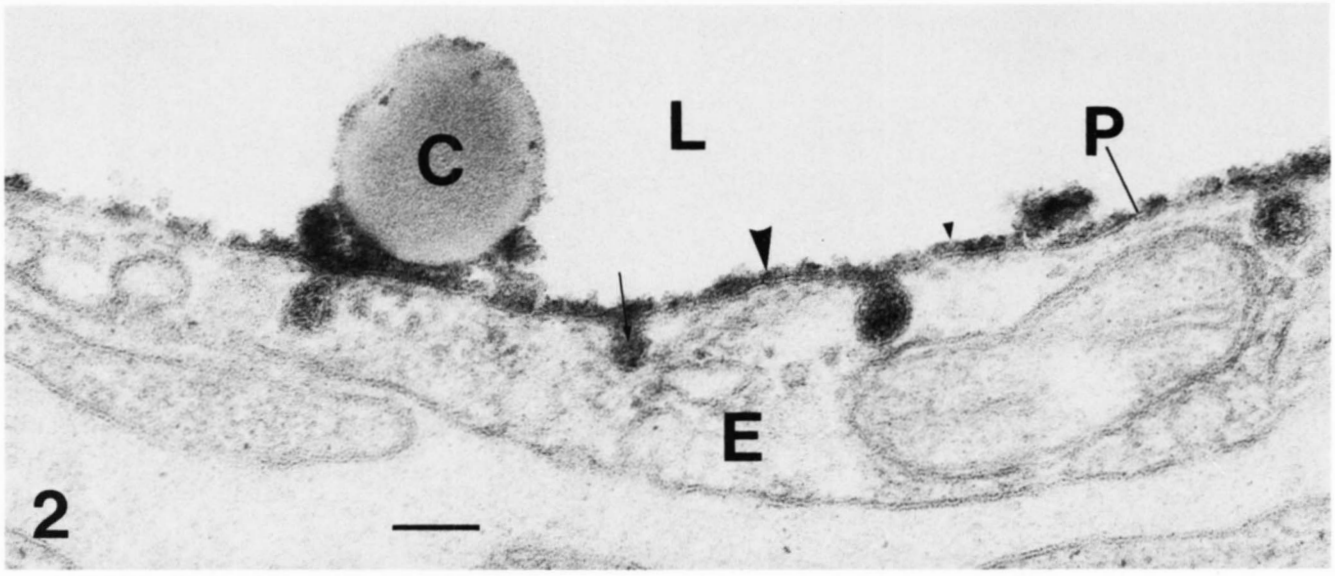

Fig. 2. High magnification electron micrograph of a portion of a heart capillary from a fat-fed rat showing indirect immunocytochemical localization of functional LPL. Immunocytochemical reaction product is present at the lumen surface of the endothelium (large arrowhead) and within an invagination (arrow). Product is also associated with a fat droplet (C) of chylomicron size situated on the lumen surface. Some electron-lucent structures in the layer of reaction product are of macromolecular size; the V-shaped structure indicated by the small arrowhead is in the size range of IgG. Same fixation, section thickness, and staining as for Fig. 1. Bar, 100 nm. Capillary endothelium (E), capillary lumen (L), plasma membrane (P).

tables, the null hypothesis that immunoreaction coverage is independent of functional LPL activity is rejected. It is concluded that an association exists between coverage and activity. From inspection of the contingency tables, that association is positive.

When the reaction product on capillary lumen surfaces exceeded 20% coverage, then, in surface profile locations where product was present in high concentrations, its structural nature was more or less obscured by image superimposition effects. Simple or complex electron-lucent structures were visible sometimes in association with reaction product (Fig. 2).

In the glucose- and fat-fed groups, the amounts of immunocytochemical product varied greatly in different parts of heart tissue blocks. Some parts contained numerous capillaries with more or less of the reaction product; in other parts, product was entirely absent. In capillaries visible in considerable lengths in sections, great differences in concentrations of product occurred occasionally along lumen surfaces. Such great differences were evident sometimes on opposite lumen surfaces of a long capillary profile. For hearts perfused with preimmune IgG instead of primary step anti-LPL IgG, and for hearts preperfused with heparin prior to immunocytochemistry, extensive searches beyond the 100 capillary samplings were made to try to locate any cap-

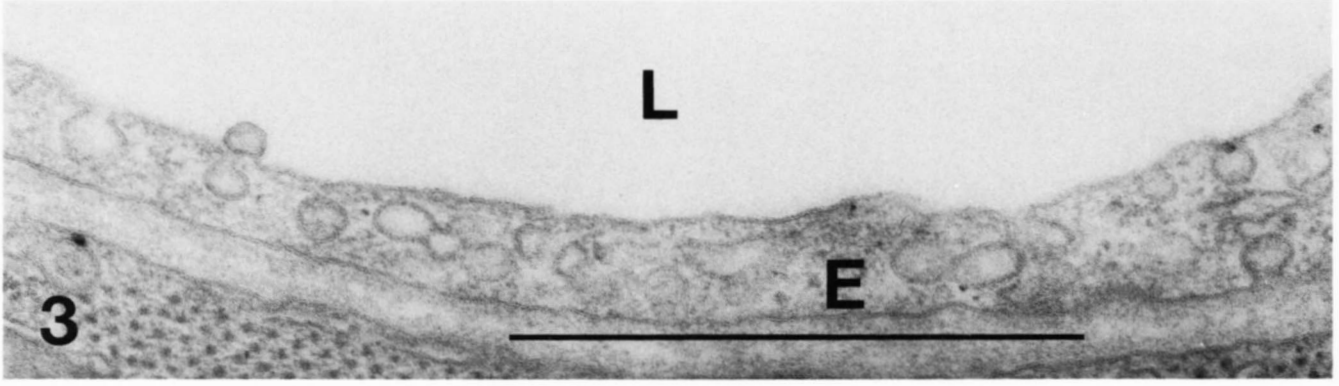

Fig. 3. Electron micrograph of a portion of heart capillary endothelium from a glucose-fed rat. Fixation, section thickness, and staining as for Fig. 1. Bar, 1 μm. Capillary endothelium (E), capillary lumen (L).

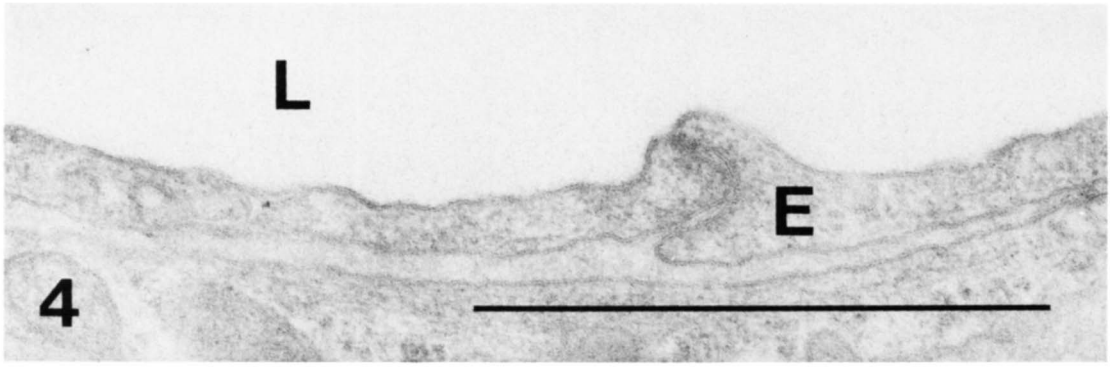

Fig. 4. Portion of heart capillary from a fat-fed rat. As a control, this heart was perfused with preimmune serum IgG instead of primary step antiLPL IgG. The lumen surface lacks immunocytochemical product. Fixation, section thickness, and staining as for Fig. 1. Bar, 1 μm. Capillary endothelium (E), capillary lumen (L).

illary profiles with product. When such capillaries were found, product was generally very low.

Characterization of anti-LPL IgG

Elution at pH 2.8 of that part of partially purified LPL which bound to anti-LPL IgG-Sepharose 4B yielded fractions without lipase activity despite immediate alkalinization. However, most of the protein was eluted in a single fraction and showed one major band on SDS polyacrylamide gel electrophoresis.

DISCUSSION

LPL is known to exist at several sites within tissues (13), but only the LPL activity released by perfusion with heparin appears to function in the uptake of circulating triglycerides (1, 2). This so-called functional LPL activity can be altered rapidly by feeding various nutrients to the rat. Functional LPL activity is high in hearts of fasted rats and increases as much as 50% several hours after fat-feeding (10) or declines as much as 80–90% several hours after glucose-feeding (11). Thus, if fasted rats are fed either fat or glucose, the hearts of the fat-fed rats reach levels of functional LPL activities ranging from 8 to 10 times the levels in hearts of the glucose-fed group. This difference is attributable to changes in the number of functional LPL molecules, estimated by immunotitration (29), and is reflected physiologically in corresponding changes in capacities of the isolated hearts to hydrolyze perfused chylomicron triglyceride (10).

From these observations we inferred that a positive association should exist between levels of functional heart LPL activities and amounts of LPL protein that might be localized immunocytochemically at the putative functional site, the lumen surface of capillary endothelium. In fact, such a positive association is dem-

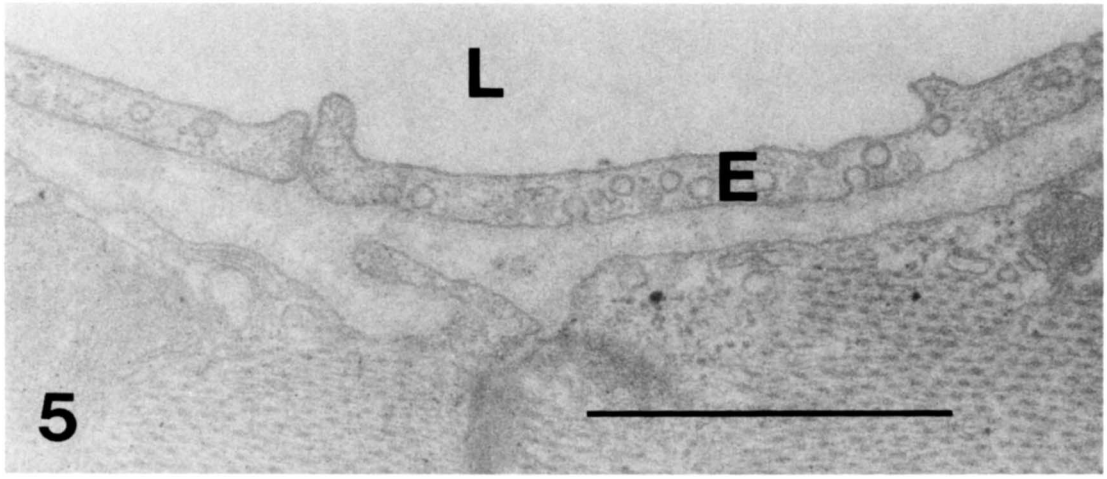

Fig. 5. Part of a capillary from a fasted rat. The heart was perfused with heparin to remove functional LPL prior to perfusion with immunocytochemical reactants. There is no immunocytochemical product on the lumen surface. Fixation procedure 1; section thickness 40 nm; section stained 1 min with alkaline lead citrate. Bar, 1 μm. Capillary endothelium (E), capillary lumen (L).

TABLE 1. Comparison of effects of heparin preperfusion or glucose- or fat-feeding on heparin-releasable LPL activity and immunocytochemical reaction for LPL in isolated rat hearts

Condition	LPL Activity[a]	Immunocytochemical Reaction	Capillary Frequency	
			Observed[c]	Expected[d]
	mU	% Coverage[b]		
Heparin preperfusion[e]	21[f]	0–20	95	95.0
		20–40	3	3.0
		40–60	1	1.4
		60–80	0	0.5
		80–100	1	0.1
Glucose-fed	118[g]	0–20	77	77.0
		20–40	20	20.0
		40–60	3	3.0
Fat-fed	994[g]	0–20	18	17.9
		20–40	14	14.0
		40–60	17	19.3
		60–80	27	24.6
		80–100	24	24.1

[a] Estimated as LPL activity released by 30 sec perfusion of an isolated heart with 1 IU heparin/ml. Hearts used for LPL activity assays could not be used for immunocytochemistry and vice versa.

[b] Percent coverage of capillary lumen surfaces by immunocytochemical reaction product. For the observed capillary frequencies only, each of the class intervals of % coverage does not contain the indicated upper limit.

[c] Data represent one heart per condition, with sample size of 100 capillaries.

[d] Calculated as 100 times the relative area (= relative frequency) under a fitted, empirical, continuous, distribution curve within the indicated class interval of % coverage. See Appendix for details.[6]

[e] Two-min heparin (1 IU/ml) preperfusion of heart isolated from fasted rat.

[f] 20.9 ± 1.8 mU (mean ± SEM, n = 7).

[g] Single determination.

onstrated in the present study with isolated hearts in which three very different levels of functional LPL activities have been experimentally induced. Although immunological specificity of the immunocytochemical primary step anti-LPL IgG is unproven, it does inactivate the functional fraction of LPL in vivo (3) and in vitro (4). Further, if components other than LPL on the lumen surface are responsible to a significant extent for some of this reaction product, then it is expected that they would be positively correlated with functional LPL

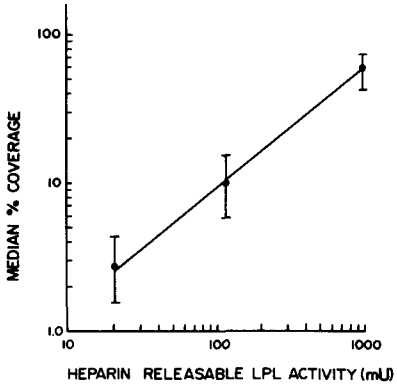

Fig. 6. Demonstration in isolated rat hearts of the positive association between median % coverage of lumen surfaces of capillaries by immunocytochemical reaction product for localization of LPL and LPL activity releasable by 30 sec perfusion with heparin (1 IU/ml KRB). Each of the three plotted points represents comparable isolated hearts, one heart for estimation of median % coverage, the other(s) for estimation of functional LPL activity. Values of median % coverage are estimated from equations for areas under continuous distribution curves fitted to the three sets of grouped data in Table 1. The 99.9% confidence interval covering the population median % coverage is indicated in each instance by the limits of a vertical line. The regression equation for the straight line minimizes the sum of the absolute vertical distances between the plotted points and the line.

TABLE 2. Test for independence between extent of immunocytochemical reaction for LPL and heparin-releasable LPL activity in the isolated heart[a]

LPL Activity[b]	Capillary Frequency of Immunocytochemical Reaction, % Coverage 0–20	20–100	G_{adj}[c]	*P* Value[d]
mU				
994	18	82	74.07	⩽0.001
118	77	23		
118	77	23	14.17	<0.001
21	95	5		

[a] Frequency data from Table 1 arranged into contingency tables, with immunoreaction coverages ≧ 20% merged into a single category.

[b] Conditions corresponding to 994, 118, and 21 mU are fat-fed, glucose-fed, and heparin preperfusion, respectively, as described in Materials and Methods.

[c] In the G-test for independence, G_{adj}-values were calculated with Williams' correction for 2 by 2 contingency tables.

[d] Based on entry of G_{adj}-values into chi-square table with one degree of freedom.

activity and, like that activity, not only responsive in a quantitatively similar way to fat- or glucose-feeding, but also susceptible to removal by heparin perfusion. Nonfunctional LPL or a tightly bound specific carrier for functional LPL might meet these criteria, but that components unrelated in their functions to the presence or function of LPL would meet all such criteria seems remote. Thus, the evidence supports the concept of a component, at capillary lumen surfaces, that not only displays LPL activity but also antigenicity responsible for at least some of the immunocytochemical reaction.

The reason for the uneven distribution of capillaries with immunocytochemical reaction product in hearts isolated from fat- or glucose-fed rats is unclear. In our electron microscopic examinations of tissue sections, all capillaries were distended because of the perfusion-fixation and a red blood cell was rarely seen. It is possible that during perfusion the glutaraldehyde fixative could have induced vascular constrictions (cf. 21) that might have impeded an even distribution of subsequently perfused 3,3′-diaminobenzidene substrate and osmium tetroxide solutions. Indeed, after perfusions of the latter two fluids, browning or blackening of the heart tissue was usually uneven and samplings were restricted to the darkened zones. Alternatively, functional LPL may in fact be unevenly distributed, not only over short distances along a capillary profile, but also among zones of capillaries in heart tissue.

The presence of immunocytochemical reaction product within lumenal plasma membrane invaginations was probably not due to trapping of unbound primary or second step immunoreactants, since product was not seen in invaginations when preimmune IgG was substituted for primary step antibody. A vesicular profile containing product may actually be part of a rosette-like invagination complex but appear isolated because of the direction of ultrathin sectioning. If any of the vesicular profiles with product represent true vesicles, then they must have been plasma membrane invaginations that pinched off prior to glutaraldehyde perfusion of the isolated heart. Vesicular profiles containing product were always observed to be near the lumen side of capillary endothelium, indicating that they probably originated from, or were extensions of, lumenal invaginations.

Chylomicron-like lipid particles remaining in the capillaries were adjacent or close to the endothelium. In the latter location these particles were presumably in contact with the endothelium at other levels in the tissue block. When immunocytochemical product was present in capillaries, it was generally present around the peripheries of these chylomicron particles as well. One interpretation for this phenomenon is that the flow of blood in capillaries brings some chylomicrons into rolling contact with endothelial glycocalyx; as each such chylomicron rolls to a halt, some LPL molecules of the glycocalyx adhere around the surface of the chylomicron while others serve as bridges across the short space between the endothelium and the now stationary chylomicron. This interpretation is compatible with the concept suggested by Olivecrona et al. (30) that LPL molecules on long flexible elements of the endothelial glycocalyx may be situated at some distance from the endothelial plasma membrane base of the glycocalyx.

In conclusion, this immunocytochemical study provides visual evidence for the location of the functional fraction of LPL at the lumen surface of the capillary. Previous light microscopic histochemical attempts to localize LPL, based on detection of free fatty acids released during enzymatic hydrolysis, lacked specificity for LPL and could not have localized any vascular LPL uninvolved in hydrolysis of chylomicrons or very low density lipoproteins. In this study nutritional and other experimental manipulations, producing large differences in heart LPL activities, permitted detection of a positive association between functional LPL activity and amounts of visible immunoreaction product on the lumen surfaces of heart capillaries.

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