Low Density Lipoprotein Transport Across a Microvascular Endothelial Barrier After Permeability Is Increased

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We investigated the pathways for low density lipoprotein (LDL) transport across an endothelial barrier in individually perfused microvessels before and after an increase in permeability. The divalent cation ionophore A23187 (5 \(\mu\)M) was used to increase microvessel permeability. LDL permeability coefficients (\(P_{S_{LDL}}\)) were measured using quantitative fluorescence microscopy. In the control state, \(P_{S_{LDL}}\) measured after 10–23 minutes of accumulation of fluorescent-labeled LDL outside the microvessel wall was \(4.8 \times 10^{-8}\) cm/sec. The transvascular vesicular exchange of approximately 50 vesicles/sec would account for the measured flux. The flux of LDL across the microvessel wall increased as much as 170-fold at the peak of the permeability increase (2–4 minutes after ionophore infusion). Permeability returned toward control values 10 minutes after ionophore infusion but remained elevated for as long as ionophore was present in the perfusate. The effective \(P_{S_{LDL}}\) was similar in magnitude to the \(P_s\) for fluorescent-labeled dextran (MW 20,000) when permeability was increased. To investigate the nature of pathways for LDL in the high-permeability state, \(P_{S_{LDL}}\) was measured at a series of microvessel pressures. LDL transport increased as microvessel pressure increased, demonstrating coupling of LDL flux to transvascular water flow. Solvent drag accounted for more than 95% of the increased flux of LDL in the period 2–10 minutes after permeability increased. Our results conform to the hypothesis that porous pathways between adjacent endothelial cells contribute to LDL transport across an endothelial barrier when permeability is increased. (Circulation Research 1990;66:486–495)

Low density lipoprotein (LDL) is internalized and degraded by normal endothelium by LDL receptor–mediated processes. Vesicular pathways are reported to transport LDL across arterial endothelium by a low-affinity uptake process. The contributions of these mechanisms to uptake and transport across microvascular endothelium of true capillary and venular microvessels remain to be determined. Furthermore, the mechanisms that promote LDL transport across the endothelial barrier when permeability is increased have not been elucidated. Previous investigations have indicated that in focal areas of increased permeability to the macromolecule horseradish peroxidase, LDL transport across aortic endothelium was also increased. In the present investigation we extended our investigations of the transport of macromolecules across the endothelial barrier in the wall of microvessels to include fluorescent-labeled LDL as a test probe.

Although morphological and physiological evidence suggests vesicular and other nonporous pathways might transport macromolecules across endothelial cells, recent evidence from experiments on isolated whole organs and individually perfused microvessels indicates that macromolecular transport may also take place through porous pathways. The proportion of transport via these extracellular pathways, which are postulated to lie between adjacent endothelial cells, increased when the permeability of the microvascular barrier was increased.

In our laboratory, methods have been developed to measure solute permeability coefficients to macromolecule transport in microvessels. To determine if LDL is transported across an endothelial barrier by extracellular porous pathways during high-perme-
ability states, we perfused individual microvessels with fluorescent-labeled LDL before and after permeability had been increased. The simplest experimental test of an extracellular pathway for LDL is the coupling of LDL flux to water flow across the microvessel wall. Because water flow increases as capillary pressure increases, we measured solute permeability coefficients to LDL at a series of capillary pressures before and after permeability had been increased with the calcium ionophore A23187.

Materials and Methods

LDL

Preparation. Human LDL from a single human source (J.C.R.) was isolated weekly by preparative ultracentrifugation \(^{12}\) and was labeled with the fluorescent hydrocarbon probe 1,1'-dioctadecyl 3,3',3' tetramethylyindocarbocyanine (DiI) as described by Pitas \textit{et al.} \(^{13}\) The labeled LDL was dialyzed against frog Ringer’s solution for 48 hours before use. LDL labeled with DiI (LDL-DiI) does not transfer the fluorescent probe because the two octadeccyl moieties make the compound extremely hydrophobic. \(^{14}\) The spectral properties of DiI are similar to rhodamine (excitation maximum, 540 nm; emission maximum, 556 nm), and the DiI does not photobleach rapidly.

Characterization. Using gradient gel electrophoresis, no difference in the electrophoretic mobility relative to front moving boundary (Rf) was found between native LDL, LDL-DiI from Dr. Pitas’ laboratory, and LDL-DiI from this laboratory. Also, using gradient gel electrophoresis, no difference in migration was noted between native LDL, LDL-DiI immediately after preparation, and LDL-DiI after 5 days of storage. A single determination of total cholesterol and total protein of the LDL-DiI yielded values of 1.16 mg/ml and 0.38 mg/ml, respectively. These values were assumed to remain constant because plasma for isolation of LDL was obtained from a single source. LDL-DiI was diluted 1:1 with frog Ringer’s solution containing bovine serum albumin (10 mg/ml) before perfusion experiments. Fluorescence intensity of a known volume of LDL in frog Ringer’s solution was measured by photometer as a function of LDL-DiI concentration over a range of 1.16–0.23 mg/ml cholesterol. A linear correlation was found with a correlation coefficient of 0.99.

Superfusate Solution

The frog Ringer’s solution had the following milimolar composition: NaCl 111, KCl 2.4, MgCl \(_{2} \) 1.0, CaCl \(_{2} \) 1.1, NaHCO\(_{3} \) 0.195, glucose 5.5, and [N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES)]-Na-HEPES 5.0. All chemicals were purchased from Mallinckrodt, St. Louis, Missouri, except HEPES acid and Na-HEPES (Research Organics, Cleveland, Ohio). The pH was adjusted to 7.4–7.45 with 0.115N NaOH. The frog Ringer’s superfusion solution was cooled to 17–20°C.

Test Solutes and Ionophore

Dextran (MW 20,000) labeled with fluorescein isothiocyanate (D20-FITC) was obtained from Pharmacia, Piscataway, New Jersey. The spectral properties of D20-FITC are excitation maximum of 489 nm and emission maximum of 515 nm.

The calcium ionophore A23187 (Calbiochem, La Jolla, California) was dissolved in ethanol (Gold Sheid Chemical, Hayward, California) to a stock solution of 5 mM, kept at 4°C, and protected from light. Immediately before microperfusion, A23187 was diluted in frog Ringer’s solution containing albumin (10 mg/ml; Sigma Chemical, St. Louis, Missouri) to a concentration of 5 \(\mu\)M. The ethanol concentration of the microperfusion solution was 0.2% (16 mM).

Animals

All in vivo experiments were performed on male leopard frogs, \textit{Rana pipiens} (6.5–7.5 cm long), supplied by J.M. Hazen, Alberg, Vermont, that were housed at 15°C. Frog mesenteric microvessels were used because 1) LDL transport can be measured across a single layer of endothelium without the confounding problems of serial barriers in the media and vasa vasorum of larger vessels, 2) extensive experience has been developed in this laboratory with the measurement of permeability properties of frog mesenteric vessels, and 3) microvessels can be cannulated easily in this animal model.

Microperfusion Techniques

General. The technique of cannulation and perfusion of microvessels used in this laboratory has been described previously. \(^{10,11}\) Briefly, the frog was pithed and positioned on a cork-lined tray. A flank incision was made, and the mesentery was floated out of the abdominal cavity and draped over a quartz pillar centered on the tray. Frog Ringer’s solution was dripped over the mesentery throughout the entire experiment. The mesentery was viewed using a Leitz (Overland Park, Kansas) inverted microscope equipped for transillumination and epi-illumination. Each arm of a Y-shaped branch of a postcapillary venule, identified by converging flow, was cannulated with micropipettes. The washout micropipette contained a clear solution of frog Ringer’s solution containing albumin (10 mg/ml), and the test pipette contained the fluorescent-labeled solute in the same albumin–frog Ringer’s solution. The microvessel could be perfused alternately with either the clear albumin solution or the labeled solution. The microvessel was perfused with the fluorescent-labeled solute for short (20–60 seconds) intervals sufficient to measure the initial flux of the test solute across the microvessel wall from the accumulation of the test solute outside the vessel. The test solute was then washed out of the vessel window by switching back to the washout perfusion. After the test solute diffused out of the window, additional measurements...
of solute permeability coefficient were made on the same segment of the microvessel wall.

Fluorescence intensity was measured by aligning the microvessel lumen and the surrounding tissue within an adjustable measuring window so that the microvessel lumen was positioned along the longitudinal axis of the measuring window and therefore lay entirely in the measuring window. The measuring window was generally two to three microvessel diameters wide and four microvessel diameters long. The image of the microvessel and surrounding tissue was projected onto a photomultiplier tube. The field was also viewed with a low-light television camera and a television screen. During perfusion of the microvessel with the labeled solute, the pressure in the washout micropipette was adjusted so that the fluorescent solute neither advanced nor receded into the pipette. This pressure was recorded as the microvessel pressure.

**Measurement of Permeability**

Permeability coefficients were calculated from the change in fluorescence intensity in the measuring window after the microvessel was filled with fluorescent solute and from the initial step change in fluorescence intensity as the test solute filled the microvessel lumen.

\[
Ps = \frac{1}{\Delta I_0} \left( \frac{dI}{dt} \right)_0 r/2
\]

Here, \( \Delta I_0 \) is the initial step increase in fluorescence intensity, \( \left( \frac{dI}{dt} \right)_0 \) is the initial rate of increase in fluorescence intensity as solute begins to accumulate outside the vessel, and \( r \) is the vessel radius.\(^{10,11}\) Values of \( Ps \) calculated from Equation 1 overestimate the true diffusive permeability when solvent drag transport contributes to the flux of solute across the microvessel wall.\(^{15}\) Thus, the value of \( Ps \) is referred to as an apparent permeability coefficient when its value increases as capillary pressure increases. The relation between \( Ps \), the permeability coefficient, and the solvent drag component of transport is described in the “Appendix.”

**Protocols**

**Protocol 1.** To compare LDL transport with a reference macromolecule, which lacks receptor-mediated pathways of transport, we perfused isolated microvessels with a perfusate containing both labeled LDL-DiI and D20-FITC. By changing narrow band light filters, we measured solute permeability coefficients of both LDL and dextran within an interval of 20–60 seconds in the same microvessel under identical experimental conditions. Measurements of solute permeability coefficients for both solutes were made at the same microvessel pressure. Measurements were made in the control state and after the permeability of the microvessel wall was increased by continuous infusion of the ionophore A23187 via the washout micropipette.

To discriminate between LDL-DiI and D20-FITC fluorescence when both solutes were in the perfusate, a series of measurements were made in which the concentrations of both solutes were adjusted to reduce the extent to which the fluorescence from DiI-labeled LDL could be detected, using the Leitz filter cube to measure D20-FITC. The test concentrations chosen were 0.58 mg/ml cholesterol and 0.19 mg/ml protein for LDL-DiI and 0.25 mg/ml for D20-FITC. At these two concentrations there was no overlap of fluorescence from FITC into the DiI (rhodamine filter cube) measurement and less than 6% overlap of DiI fluorescence into the dextran (fluorescein filter cube) measurement.

**Protocol 2.** To partition the transvascular flux into diffusive and convective components, we perfused individual microvessels in the control state and measured the change in flux at microvessel pressures between 3 and 20 cm H\(_2\)O. Then permeability was increased by exchanging the washout micropipette with one containing A23187. During infusion of A23187, \( Ps \) for LDL was measured at 2-minute intervals over the same range of microvessel pressures.

**Results**

**Transmicrovascular Transport of LDL and High Molecular Weight Dextran**

Addition of the calcium ionophore to the perfusate resulted in a large transient increase in the transvascular flux of fluorescently labeled LDL. In seven vessels, the permeability coefficients of both LDL-DiI and D20-FITC were measured in the same vessel by using protocol 1. Figures 1A and 1B show the
response for all seven vessels with microvessel pressures in the range of $8-10 \text{ cm H}_2\text{O}$. Mean values are pooled from individual experiments from experimental points with 2-minute time intervals. Because of the rapid changes in $P_s$, there was not sufficient time to measure both dextran and LDL within the first 2 minutes of the transient. All data show a transient increase in permeability to a peak 2–4 minutes after the addition of the ionophore and return toward, but do not reach, control values over a period 6–10 minutes after the initiation of A23187 infusion. In all vessels, permeability remained elevated for as long as the ionophore was in the perfusate. We measured permeability coefficients during the sustained phase of permeability increase for up to 18 minutes after adding ionophore.

The striking feature of the transient increase in permeability coefficients for both solutes is that the magnitude of the apparent permeability coefficient at the peak of the response was similar for both solutes although LDL and dextran differ in molecular size and chemical composition. The mean ± SEM values of the apparent permeability coefficients to LDL and dextran at the peak of individual transients in permeability were $96.2 \pm 15.6 \times 10^{-7}$ cm/sec and $114.2 \pm 15.7 \times 10^{-7}$ cm/sec, respectively.

We carried out additional experiments to demonstrate that the presence of LDL did not modify the magnitude and time course of the transient increase in D20 permeability coefficients. This result indicates that there was no significant binding of A23187 by LDL, and no modification of the structure of the pathway for D20 when LDL was in the perfusate. We also demonstrated that the perfusate containing the vehicle used to solubilize the ionophore (ethyl alcohol, 0.2%) without A23187 produced no significant increase in permeability when compared with control perfusion (eight microvessels). In three additional experiments, we used the ionophore at a concentration of 1 μM instead of 5 μM. The time course in each vessel was similar to that for LDL in Figure 1A, but the magnitude of the peak response was reduced. For LDL, the apparent permeability coefficient at peak response was $58 \pm 23 \times 10^{-7}$ cm/sec when the ionophore concentration was 1 μM.

These experiments suggest that similar mechanisms are responsible for the transport of both solutes across the endothelial barrier during the high-permeability state produced by addition of the ionophore. The mechanisms that account for this flux are explored in the experiments described in the next section.

**Coupling of LDL Transvascular Flux to Transvascular Water Flow**

Figure 2 is an example of a single experiment as described in protocol 2. We measured transvascular flux for at least two different microvessel pressures at 2-minute intervals during the transient. In the experiment of Figure 2, we measured the slope in four of six time intervals. In the other nine vessels, we obtained slopes and intercepts from four intervals in one vessel, three intervals in two vessels, and two intervals in six vessels. No estimate could be obtained if transvascular flux measurements were not successful for at least two pressures during a 2-minute interval. Our protocol meant that a single experimental hitch, such as a leak of washout solution into the capillary lumen while measuring balance pressure or a failure to rapidly fill the microvessel lumen with test solute at the beginning of the permeability determination after washout, resulted in loss of data for a particular time interval. The slope of the linear relation between apparent permeability and microvessel pressure was greatest during the interval 2–4 minutes after addition of the calcium ionophore to the perfusate of the transient. The intercept of the linear relation between apparent permeability and microvessel pressure on the permeability axis, $P_{0(0)}$, is an estimate of the true permeability coefficient measured when water flow across the vessel wall is close to zero.

Table 1 is a summary of the data from all 10 vessels studied using protocol 2. The slopes of the regression of permeability on microvessel pressure are given (where measured) at 2-minute intervals after A23187 infusion (in part A). According to the analysis given in the “Appendix,” the slope is a measure of the membrane permeability parameters that determine solvent drag mechanisms and is designated $L_m(1-\sigma)$. $L_p$ is the hydraulic conductivity of the water pathways in which LDL can pass and $1-\sigma$ is a solvent drag or coupling or sieving coefficient in these pathways. The mean slopes are significantly different from control ($p<0.05$) for the time intervals 2–4 and 4–6 minutes after ionophore infusion (Figure 3). The slopes are not significantly different from control after 6 minutes. Estimates of $P_{0(0)}$ are given in part B of Table 1. Negative values of $P_{0(0)}$ have no physical meaning. They reflect our inability to account for the nonlin-
TABLE 1. Slopes and Intercepts of the Regression of $P_{S_{LDL}}$ on Microvessel Pressure Measured at 2-Minute Intervals

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Time interval (min)</th>
<th>A. Slope</th>
<th>B. Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2–4</td>
<td>4–6</td>
<td>6–8</td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.6</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.2</td>
<td>-0.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>8</td>
<td>1.7</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>9</td>
<td>1.3</td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>8.0</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>3.74±1.4</td>
<td>2.22±0.40</td>
<td>2.27±0.79</td>
</tr>
</tbody>
</table>

Slope (part A) is an estimate of the membrane coefficients determining solvent drag, $L_p(1-\sigma)$ cm/sec cm H$_2$O × 10$^7$. Intercept (part B) is the permeability coefficient at zero volume flow, $P_0$ cm/sec×10$^7$. $P_{S_{LDL}}$, low density lipoprotein apparent permeability coefficient.

The control experiments for the above studies, we measured the dependence of permeability coefficients on microvessel pressure when there was no ionophore in the perfusate. In these experiments, $P_{S_{LDL}}$ was measured after 30 seconds of solute accumulation. For LDL, the mean value of the apparent permeability in all 17 vessels using protocols 1 and 2 was 2.1±1.25×10$^{-7}$ cm/sec at a mean pressure of 9.6 cm H$_2$O. In 11 of the 17 vessels used for experiments with protocols 1 and 2, the mean slope of the relation between apparent permeability and microvessel pressure was 0.1±0.52×10$^{-7}$ cm/sec for each cm H$_2$O increase in microvessel pressure. In other vessels, apparent permeabilities were measured at only one pressure or at two pressures that differed by less than 3 cm H$_2$O. The slope of the relation between apparent LDL permeability and microvessel pressure for control experiments was not significantly different from zero.

The control values for $P_{S_{LDL}}$, of the order of 2×10$^{-7}$ cm/sec, measured over a time period of 30 seconds, were up to 10-fold larger than values of $P_{S_{LDL}}$ measured in arterial endothelium over time periods of 30–60 minutes$^{16-18}$ (Table 2). To compare our results with published values, we extended the period of time over which $P_{S_{LDL}}$ was measured to

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Values (mean±SEM) for the coefficients determining solvent drag [Lp(1-\sigma)] across the microvessel wall during 2-minute intervals from experiments shown in Figure 2 (data in Table 2).
10–23 minutes. The mean LDL permeability coefficient for these long-term experiments was \(0.48 \times 10^{-7}\) cm/sec. This value was less than one fourth of those measured over the shorter interval. In contrast, short (30-second) and long (6–22-minute)-term measurements of Ps for dextran in three microvessels were not significantly different (short-term, \(3.2 \pm 2.9 \times 10^{-7}\); long-term, \(2.0 \pm 1.8 \times 10^{-7}\) cm/sec).

The fourfold difference in apparent permeability coefficients between short- and long-term measurements for \(P_{s,LDL}\) could not be explained by losses of LDL from the measuring window. This was because the longer term accumulation of dextran (MW 20,000), a smaller, more rapidly diffusing molecule than LDL, led to estimates of Ps that were the same as values for short-term dextran experiments. If loss of tracer was the source of the lower values of Ps in the long-term experiments, then the Ps for dextran should have been reduced more than Ps for LDL. However, the larger apparent permeability to LDL for short-term measurements might be explained if uptake and binding of LDL by the endothelial cell occurred during the first 30 seconds of the measurement. Once saturated, this process would contribute less to LDL accumulation. To test this idea, we measured control LDL permeability coefficients in three microvessels using unlabeled LDL in the washout micropipette at the same concentration as in the test micropipette. Three measurements were made on each microvessel: 1) with no unlabeled LDL in the washout micropipette, 2) with unlabeled LDL in the washout micropipette, and 3) repeat of 1). The corresponding values of \(P_{s,LDL}\) were (in \(10^{-7}\) centimeters per second): \(3.2 \pm 0.7, 0.6 \pm 0.6\), and \(2.6 \pm 1.6\). These results show that prior exposure of the microvessel wall to unlabeled LDL reduced the measured \(P_{s,LDL}\) in the short-term experiment to a value similar to that measured in long-term experiments.

To check that initial LDL binding and uptake did not affect the response to the ionophore, we added unlabeled LDL to the washout and repeated measurements of \(P_{s,LDL}\) in the control state and after adding ionophore to perfusate. In three experiments, \(P_{s,LDL}\) increased from \(0.88 \pm 0.53 \times 10^{-7}\) cm/sec to a peak of \(97.3 \pm 14.6 \times 10^{-7}\) cm/sec 2–4 minutes after addition of the ionophore. \(P_{s,LDL}\) measurements performed after A23187 infusion were made from 30-second LDL perfusions. \(P_{s,LDL}\) measurements after infusion of the ionophore were made from 15–30-second LDL perfusions. The time course of the permeability change was the same as in Figure 1.

**Discussion**

The results of our experiments conform to the hypothesis that, in the high-permeability state, large porous pathways, presumed to lie between adjacent endothelial cells, contribute to the transport of LDL across the endothelial barrier. Our observations that during the transient increase in permeability, the magnitude of the apparent permeability coefficient for LDL is 1) similar to that of a neutral dextran (D20) and 2) proportional to microvessel pressure and hence transvascular water flows enable further quantitative evaluation of the nature of the transport mechanisms responsible for LDL flux when permeability is increased. Before developing these arguments, we will evaluate our results for measurements of Ps for LDL in the normal state.

**LDL Transport in Control Microvessels**

Table 2 compares the apparent permeability coefficients for LDL measured after 10–23 minutes of accumulation outside microvessels in our experiments with estimates of \(P_{s,LDL}\) in rabbit arterial wall after 30 and 60 minutes of accumulation. The values of the effective permeability coefficients for LDL across the rabbit arterial wall alone are approximately one half the value measured in our frog mesenteric microvessel experiments.

Our control measurements are consistent with the hypothesis that in microvessels with intact endothelial barriers, LDL crosses the endothelium first by weak binding and uptake into lumenal vesicles and then by transport via a vesicular system. Uptake of a single fluorescent-labeled LDL into approximately 2,000–4,000 vesicles per endothelial cell (≈10% of the luminal population of vesicles in frog mesenteric vessels) during the first 30 seconds of perfusion would be measured as an effective permeability of \(2–5 \times 10^{-7}\) cm/sec in a microvessel of 15 \(\mu\)m radius, containing LDL at the concentration used in our experiments (≈0.35 \(\mu\)M). This figure is not a true permeability coefficient because at these low levels of permeability this study design does not distinguish between uptake into the endothelial cells and transport across the cells. The reason is that changes in fluorescence intensity were measured over an area encompassing the microvessel and surrounding tissue. To account for the measured transport of LDL over a 10–20-minute period, approximately 50 vesicles would have to exchange a labeled LDL across each endothelial cell each second. Although our data are consistent with a vesicular transport mechanism, we should note that significant binding of LDL to the wall during the first 30 seconds of an LDL perfusion may mask the contribution of a porous pathway to LDL exchange.

LDL, when used as a test probe, has a number of advantageous properties for the study of macromo-

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**Table 2. Comparison of Effective Permeability Coefficients for Low Density Lipoprotein Transport Under Control Conditions**

<table>
<thead>
<tr>
<th>Endothelial barrier</th>
<th>(P_{s,LDL}) (cm/sec (\times 10^9))</th>
<th>Time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit arterial wall</td>
<td>2.7</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>Rabbit arterial wall</td>
<td>1.95</td>
<td>30 and 60</td>
<td>17</td>
</tr>
<tr>
<td>Rabbit arterial wall</td>
<td>1.85</td>
<td>30 and 60</td>
<td>18</td>
</tr>
<tr>
<td>Rabbit arterial wall (intima only)</td>
<td>0.67</td>
<td>30 and 60</td>
<td>18</td>
</tr>
<tr>
<td>Frog mesenteric microvessels</td>
<td>4.8</td>
<td>10–23</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(P_{s,LDL}\), low density lipoprotein apparent permeability coefficient.
lecular transport in this and future studies. LDL has a large effective radius, which allows comparison with smaller solutes. The spectral properties of LDL-Dil enable us to examine two or more fluorescent-labeled solutes of different spectral properties simultaneously. Additionally, at least three pathways for LDL are reported in the literature: receptor-mediated, \(1^\text{st}-3\) vesicular, \(4^\text{th}\) and large pore. \(19-21\) By manipulating the experimental conditions, the relative contribution of a single pathway can be determined. Finally, LDL deposition in the arterial wall is reported to be an important component of atherogenesis. \(22\) Our findings suggest that LDL can reach the intima via large pores between endothelial cells in a high-permeability state.

In these experiments it is unlikely that high-affinity receptor-mediated uptake is a major pathway of accumulation of LDL into the microvessel wall. Our experiments demonstrated that the change in permeability coefficient in response to calcium ionophore was similar to that of dextran, which lacks receptor-mediated uptake. Studies performed in endothelial cell cultures at confluence \(4\) and in whole arteries \(18\) indicate high-affinity receptor-mediated uptake accounts for a small minority of total transport. Also, it is unlikely that human LDL will interact with high-affinity receptors on frog endothelium.

These experiments extend our methods to measure the permeability properties of individually perfused microvessels using quantitative fluorescence microscopy. The methods have been described and critically evaluated in several recent publications. \(10,11\) An important new part of our methodology is the demonstration that our method is sufficiently sensitive to measure low-affinity initial binding and uptake of LDL. This binding and uptake can be investigated directly using labeled and unlabeled tracer. Although the types of experiments described in this paper may eventually prove of value to investigations of LDL transport in large vessels, our present focus is on single microvessels, in which the endothelial layer and its associated basement membrane are the primary barrier to transport. The complications of serial barriers in the media and of additional transport of LDL into the vessel wall via the vasa vasorum are thereby avoided.

Our methods provide the most direct way to investigate LDL transport under controlled conditions in an intact endothelial layer. The permeability properties of the frog mesentery barrier to a wide range of test solutes is well documented, \(9,11\) and we chose this endothelial barrier as the starting point for our investigations. Preliminary studies performed in this laboratory to measure the flux of fluorescent-labeled albumin across the walls of hamster venular microvessels showed similar control and ionophore-induced permeability properties as were measured for dextran in this study. \(11,23\)

**Ionophore-Induced Permeability Increase**

We have demonstrated that the permeability of frog microvessels to albumin and water is increased after the addition of the divalent ionophore to the perfusate. \(11,24\) The time course of permeability changes is similar to that found in the present experiments for LDL. There is a peak in the permeability increase 2–4 minutes after addition of ionophore; permeability then returns toward control but remains elevated for as long as ionophore is present. Other laboratories have also reported reversible increases in permeability using A23187 in frog microvessels. \(25,26\) Clough and Michel \(27\) examined the ultrastructure of the same vessels as used in our study after treatment with A23187 and demonstrated gaps between endothelial cells and fenestrations in the vessel wall 5–10 minutes after application of A23187 (5 \(\mu M\)). These structures are not present in normal vessels; there is, therefore, a change in the microvesSEL ultrastructure exposed to ionophore to resemble the classical appearance of gaps between endothelial cells in postcapillary venules after exposure to an inflammatory mediator. Our experiments enable the transport of LDL across the wall during this high-permeability state to be examined.

We are aware that the use of this calcium ionophore may produce many other responses in microvessel endothelial cells (increased prostaglandin production, release of endothelial cell relaxing factor, and superoxide ion production). All or some of these responses may modulate the permeability properties of the wall that we are studying in as yet unknown ways. The utility of our approach using the ionophore lies in the ability to induce a reproducible transient permeability increase to investigate the physical and chemical natures of the pathways characteristic of a high-permeability state. We demonstrate the utility of our method in understanding further the nature of the pathways for LDL transport below.

**Solvent Drag (Convective Transport) of LDL Across the Microvascular Endothelial Cell Layer**

In contrast to the control state, the transvascular flux of LDL is strongly coupled to transversable water flow after permeability is increased. The slope of the relation between \(P_{\text{LDL}}\) and pressure is a measure of the permeability coefficients of the endothelial barrier that determine the convective transport of LDL, or solvent drag. This slope is equal to \(L_w(1-\sigma)\), as described above. The reflection coefficient, \(\sigma\), approaches a value of zero when the endothelial barrier is disrupted.

We compared the \(L_w\) of the pathways in which LDL couples to water flow with the \(L_p\) for the whole vessel wall as follows. If \(\sigma\) for LDL is assumed to be close to zero near the peak of the transient increase in permeability, then the value of \(L_p(1-\sigma)\) measured 2–4 minutes into the transient is a measure of \(L_p\) for pathways permeable to LDL. If \(\sigma\) is not zero, but is as large as 0.5, our estimates of \(L_w\) for the pathway permeable to LDL would double. From Table 1, this estimate is \(3.7\times10^{-7}\) cm/sec cm H_2O, and it is only a small fraction (7.5–15%) of the \(L_p\) of the whole vessel wall (\(20–40\times10^{-7}\) cm/sec cm H_2O) measured at the
peak of the transient increase in water conductivity. The result indicates that not all the pathways with increased water permeability have the structure modified to the extent that they are also permeable to LDL. In other words, there is a distribution of channel sizes in the high-permeability state, with LDL crossing only via the largest channels.

It should be noted that a single open gap, 0.5 μm wide and 0.5 μm long, within a segment 300 μm along a microvessel (the length of our measuring window) would impart an Lp of 10−3 cm/sec cm H2O to that segment if the gap were the only resistance to water flow. Because gap density is actually much higher,27 the expected Lp of open gaps would exceed 10−3 cm/sec cm H2O. This value is three orders of magnitude larger than the values of Lp for the pathway associated with LDL transport in this study, and it is close to two orders of magnitude more than the largest Lp s we have ever measured.9 It follows that there is a large resistance to water flow due to structures within the gap, probably associated with basement membrane and barriers with the tissue around the microvessel. These same barriers are also likely to restrict LDL transport within extracellular pathways. There is direct evidence for these barriers in the literature. For example, other large macromolecules, chylomicrons, accumulated within gaps between adjacent endothelial cells formed following exposure to inflammatory agents, presumably because of additional resistance barriers present within the gap.28

Table 1 also shows that the value of PSLDL, estimated for the limit when there is no net water flow across the vessel wall [P(0)], is 7.8×10−7 cm/sec 2−4 minutes after adding ionophore. This value is more than one order of magnitude larger than control values for PSLDL. The increased permeability probably indicates diffusion through the extracellular pathway between cells. Despite the increased diffusive permeability, the contribution of diffusion to the transport of LDL at microvessel pressures of 8−10 cm H2O is small during the early part of the transient increase in permeability because of the large contribution of solvent drag. We evaluate this below.

The relative contribution of solvent drag to the total flux is determined by the ratio Jc/S(1−σ)/P(0). Here Jc/S is transvascular water flux, and its value is equal to the product of Lp(1−σ) and capillary pressure (assuming the effective osmotic pressure of albumin in the perfusate is close to zero). Specifically, at an effective filtration pressure of 10 cm H2O, the ratio Jc/S(1−σ)/P(0) is 4.7. When this ratio is greater than 3, greater than 90% total flux is due to solvent drag. Table 3 shows that solvent drag accounts for 97.6−99.9% of total flux during the period 2−10 minutes after addition of ionophore A23187.

The above calculations explain the observation that, during the period 2−10 minutes after the ionophore infusion, Ps for LDL and D20 are of similar magnitude, even though these solutes differ in molecular size (effective radii of 20 and 4 nm, respectively). Both estimates of Ps reflect the predominant role of solvent drag during the early part of the period of increased permeability. When solvent drag is the principal transport mechanism, the value of PSDL is equal to Jc/S(1−σ). The 95% confidence limits of the peak value of Lp(1−σ) are 2−6×10−7 cm/sec cm H2O. Apparent permeability coefficient in the range of 20−120×10−7 for both solutes would be expected for microvessel pressures of 8−20 cm H2O.

As shown in the experiment in Figure 2 and summarized in Figure 3, the slope of the relation between Ps and pressure decreased with time after the peak permeability. We might therefore expect a decrease in the contribution of convective transport with time after infusion of the ionophore. After 10 minutes of perfusion with the ionophore, the contribution of convective flux to LDL transport was reduced from greater than 97% of total flux to 48.5% of total flux. Diffusive transport therefore accounts for approximately half the LDL flux in this sustained phase of the permeability increase. At this time the total flux was reduced to 6−30% of the maximal flux.

Conclusions

Our principal conclusions from the quantitative studies of LDL transport in the high-permeability state are as follows: First, the change in LDL permeability after a single stimulus produced by calcium influx into endothelial cells is transient, with a peak permeability response occurring 2−4 minutes after the stimulus and return toward control values over a period of 6−10 minutes. Second, the flux of LDL across the microvessel wall may increase as much as 170-fold at the peak of the permeability response; the mechanism responsible for the large flux across the endothelial barrier is likely to be the temporary opening of gaps between adjacent endothelial cells to form large, porous pathways. Third, over the period of 2−10 minutes after the transient increase in permeability begins, mechanisms within the microvessel wall act to increase resistance to water flow within the newly formed gaps and begin to change the
mechanism of LDL transport from solvent drag dominating to diffusion dominating (and possibly other dissipative mechanisms). It is interesting to speculate that substances which form a barrier to transport, such as a fiber matrix associated with the endothelial cell surface and basement membrane, may be produced by endothelial cells and limit the very large LDL accumulation that would occur if solvent drag remained the predominant mechanism of transport.

Transient changes in permeability are characteristic of permeability increases caused by inflammatory mediators in post capillary venules. These changes are associated with transient increases in endothelial cells’ cytoplasmic free calcium. Very few studies have been made on the early stage of permeability changes in endothelial barriers in large vessels. However, recent studies show transient changes in the ultrastructure of the endothelial barrier in aortic and femoral vessels after endotoxin infusion may produce permeability changes similar to those studied in these experiments. It remains to be determined if the mechanisms we describe are part of a characteristic endothelial cell response to repeated mild injury.

Appendix

In a water-filled channel, the total solute flux ($J_s$) crossing the microvessel wall is the sum of a dissipative component ($J_{sd}$) and a convective component ($J_{sv}$):

$$J_s = J_{sd} + J_{sv} \quad (1)$$

$J_{sd}$ is determined by a solute permeability coefficient $P_{0s}$ at zero volume flow; $J_{sv}$ is determined by the transvascular fluid exchange ($J_v/S$) and a sieving coefficient $(1-\sigma)$. Curry derived the relation:

$$J_v/S = P_{0s}(Z) + (J_v/S)(1-\sigma)C_p \quad (2)$$

where $C_p$ is plasma concentration of solute, $\Delta C$ is the solute concentration difference across the vessel wall, $S$ is surface area, $\sigma$ is the solute reflection coefficient, and $Z$ is given by the relation:

$$Z = Pe/\exp(Pe - 1), \text{ with } Pe = J_v/S(1-\sigma)/P_{0s} \quad (3)$$

We measure apparent permeability ($J_v/S/\Delta C$) under conditions where $C_p = \Delta C$. Therefore, $P_s = P_{0s}(Z) + J_v/S(1-\sigma)$. $J_s$ is determined by the Starling Relation:

$$J_s = L_p(P_e - P_t) - \sigma(\Pi_c - \Pi_t) \quad (5)$$

where $P_e$ is microvessel pressure, $P_t$ is tissue pressure, and $\Pi$ is colloid osmotic pressure. When both $P_e$ and the effective osmotic pressure across the microvessel wall [$\sigma(\Pi_c - \Pi_t)$] are small, Equation 5 reduces to the form:

$$J_v/S = L_p(P_e) \quad (6)$$

Substitution of Equation 6 into Equation 4 gives:

$$P_s = P_{0s}(Z) + L_p(1-\sigma)P_e \quad (7)$$

When $P_e = 0$, $J_v/S = 0$ and $P_s = P_{0s}$. When $P_e$ is >3, $Z = 0$ and $P_s = L_p(1-\sigma)P_e$. The slope of the relation between $P_s$ and $P_e$ is $L_p(1-\sigma)$. In all our experiments, $\sigma$ is <3.6 cm H2O.

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