Role of the Low Density Lipoprotein Receptor in Penetration of Low Density Lipoprotein into Rabbit Aortic Wall

Olov Wiklund, Thomas E. Carew, and Daniel Steinberg

The present study was designed to determine whether binding of low density lipoprotein (LDL) to endothelial LDL receptors contributes significantly to the penetration of LDL into the normal rabbit aorta. Initial flux rate was used as a measure of uptake of LDL. Reductive methylation of LDL is known to block its recognition by the LDL receptor. Therefore, the difference in flux rates of native LDL and reductively methylated LDL (methyl-LDL) was assumed to represent the receptor-dependent uptake. Native LDL and methyl-LDL were labeled with different isotopes (125I or 131I) and both were injected simultaneously into the same rabbit. After 30 to 60 minutes, trichloroacetic acid-precipitable counts were determined in aortic specimens. The initial flux rates, expressed as plasma clearance (nl/g/hr), were 1787 for native LDL and 1924 for methyl-LDL. The difference was not significant, which suggests that the flux of LDL into the aorta is not significantly dependent upon, or regulated by, endothelial LDL receptors, but is mediated by other mechanisms.

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The main characteristics of the developing atherosclerotic lesion are cell proliferation, an increase in intercellular matrix, and cholesterol accumulation. Several lines of evidence support the conclusion that most of the cholesterol originates from plasma lipoproteins, primarily low density lipoproteins (LDL). It has been shown that accumulation of immunoreactive apo B is associated with cholesterol accumulation in the plaque,1-3 and that lipoproteins very similar to circulating LDL are found in developing human atherosclerotic lesions.4,5 In experimental animals6-9 as well as in humans,10,11 a considerable penetration of labeled plasma LDL into the arterial wall has been shown. Recent studies in rabbits12 have also shown that, expressed in terms of degradation per milligram of cell protein per unit time, the intima is one of the most actively LDL-catabolizing tissues in the body.

To penetrate the arterial wall, LDL must first interact with the luminal surface of the artery and penetrate the monolayer of endothelial cells. Little is known about the mechanisms for this transendothelial transport. Most available evidence suggests that the LDL does not penetrate between endothelial cells but that flux takes place through the cells, i.e., by a transcellular vesicular transport.13,14 This vesicular transport of LDL could be via nonspecific fluid phase endocytosis, via specific LDL receptor-mediated endocytosis, or via both mechanisms. Endothelial cells in culture express a functional LDL receptor.15-19 Under conditions of tight confluency under some culture conditions, endothelial cells have reportedly suppressed essentially all LDL receptor expression.16-19 However, recent studies from this laboratory provide evidence that in the rabbit aortic intima in vivo about 40% of the LDL degradation takes place via the high affinity receptor.12 However, these results relate only to those LDL molecules that enter the endothelial cell and go on to be degraded (measured by trapping of the covalently linked tyramine...
cellulose marker). Any LDL molecules entering the cell and then exiting (either to the adventitial surface or back to the plasma compartment) without degradation would not be "seen" in such a study.

The present study was designed to investigate whether binding of LDL to endothelial LDL receptors significantly contributes to the penetration of LDL into the normal rabbit aorta. The initial overall rates of entry were measured by using conventionally iodinated native LDL injected 30 to 60 minutes before sacrifice. To assess the fraction entering via the receptor, advantage was taken of the fact that reductive methylation of LDL blocks its recognition by the LDL receptor. Reductively methylated LDL (methyl-LDL) labeled with a different isotope of radioiodine was injected simultaneously with the native LDL. The initial rates of entry of native and reductively methylated LDL did not differ significantly. Therefore, we conclude that, at most, only a very small percentage of transendothelial transport of LDL in the normal rabbit is mediated by the high-affinity LDL receptor.

Methods

LDL Preparation and Labeling

LDL (d = 1.020–1.060 g/ml) was prepared from fresh rabbit plasma containing disodium ethylenediamine tetraacetate (1 mg/ml) by differential ultracentrifugation. Reductively methylated LDL was prepared as described by Weisgraber et al. by addition of formaldehyde in the presence of sodium borohydride. Methylation was carried out before iodination.

Native LDL and methylated LDL were labeled at pH 9.5 with carrier-free 125I or 131I (Amersham) by addition of formaldehyde in the presence of sodium borohydride. Methylation was carried out before iodination.

Native LDL and methylated LDL were labeled at pH 9.5 with carrier-free 125I or 131I (Amersham) by using 1,3,4,6-tetrachloro-3,6-diphenylglycouril, a solid phase oxidant (iodogen, Pierce Chemical Company, Rockford, Illinois). Unbound iodine was removed by exhaustive dialysis against 0.15 M NaCl containing 0.01% EDTA and 20 mM sodium phosphate buffer (PBS), pH 7.4.

Animal Studies

New Zealand white rabbits (2.5–4.7 kg) were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg), and indwelling Silastic catheters were placed in the external jugular veins for injection of labeled LDL and methyl-LDL and for blood sampling. Plasma concentrations of tracer were determined in terms of trichloroacetic acid (TCA)-precipitable radioactivity in plasma. The rabbits were kept under general anesthesia during the whole experiment. At termination of the experiment, 30 or 60 minutes after LDL injection, the systemic circulation was perfused at 100 mm Hg pressure using PBS. The perfusion was continued for 5 to 10 minutes. The descending aorta was rapidly, but gently, removed and loose adventitial tissue dissected away. The descending thoracic aorta was divided into three segments each weighing 0.080–0.200 g. The aortic segments were opened longitudinally and pinned flat, and the intimal surface was gently brushed with a moistened cotton swab to remove the intima.

The cotton swabs and the brushed aortic segments were put into counting tubes and assayed for radioiodine. The aortic segments were homogenized in water, first with a Brinkman Polytron (Brinkman Instruments, Westbury, New York) and then with a motorized Teflon-glass homogenizer. The homogenates were put into counting tubes and total radioiodine was assayed. The TCA was added to a final concentration of 10% and after centrifugation, the soluble radioiodine was assayed on an aliquot of the supernate. TCA precipitation was similarly performed on an alkaline digest of the cotton swabs.

In one series of experiments, the rabbits were fed 2% cholesterol in their regular chow for 3 days before the experiments.

51Cr-Labeled Erythrocytes

51Cr-labeled erythrocytes were prepared by mixing 20 ml of fresh rabbit blood with 9 ml of a solution containing: 1.47% dextrose; 1.32% sodium citrate 0.44% citric acid; 1.0 ml ascorbic acid (75 mg/ml); and 5 mCi of 51Cr as sodium chromate (Amersham). The cell suspension was incubated at room temperature for 40 minutes with gentle intermittent stirring. The erythrocytes were then isolated by centrifugation and washed four times in 50 ml 0.155 M NaCl. The labeled erythrocytes were finally resuspended in 20 ml of 0.155 M NaCl and injected into the rabbits (about 2 x 10⁸ cpm into each rabbit).

Data Analysis

Radioassays were performed in a double-channel gamma counter, with appropriate counting windows and correction factors for simultaneous assay of 125I and 131I. All activities were corrected for physical decay. Time-averaged plasma concentrations of radioactivity during the experiments were calculated from a monoeXponential decay curve for the plasma radioactivities:

\[
\text{time-averaged concentration} = \frac{1}{t} \times \frac{C_0}{k} (1-e^{-kt})
\]  

where t = time, Co = initial concentration, and k = rate constant.

The flux of LDL or methyl-LDL was expressed as plasma clearance (nl/g aorta/hour):

\[
\text{TCA-precipitable radioactivity (cpm in tissue or tissue fraction)} \times \frac{t}{\text{time-averaged plasma radioactivity (cpm/nl)} \times \text{tissue weight (g)} \times \text{time (hr)}}
\]
Table 1. Comparison of Initial Flux of Native LDL and of Reductively Methylated LDL Into Rabbit Aorta

<table>
<thead>
<tr>
<th>LDL</th>
<th>Uptake of methyl-LDL</th>
<th>Uptake of native LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clearance (nl serum/g aorta/hr)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Into media plus adventitia</td>
<td>Into intima</td>
</tr>
<tr>
<td>Native</td>
<td>1123 ± 613</td>
<td>663 ± 337</td>
</tr>
<tr>
<td>Methyl</td>
<td>1173 ± 627</td>
<td>751 ± 373</td>
</tr>
<tr>
<td>Uptake of methyl-LDL</td>
<td>1.06 ± 0.12 (NS)</td>
<td>1.24 ± 0.42 (NS)</td>
</tr>
<tr>
<td>Uptake of native LDL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Uptake was determined at 30 minutes in three animals and at 60 minutes in seven animals. Results are means ± SD.

**Results**

**Initial Entry Rate of Native LDL and Reductively Methylated LDL Into Normal Rabbit Aortas**

The initial entry rates of native LDL and methyl-LDL were determined in 10 rabbits. In seven of these, the study was terminated at about 60 minutes after injection of the tracers (mean 62.8 minutes) and in three, at about 30 minutes (mean 32.4 minutes).

The total initial flux rates, expressed as plasma clearance (nl/g aorta/hr), were 1787 for native LDL and 1924 for methyl-LDL (Table 1). The differences in entry rates for native LDL and methyl-LDL were not statistically significant. Of the total LDL entering the aortic wall, 37% was recovered from the intimal layer in the case of native LDL and 39% in the case of methyl-LDL.

Since methyl-LDL is not recognized by the LDL receptor, its FCR is lower than that for native LDL.24 Even during the first 30 to 60 minutes in the present studies, methyl-LDL showed a significantly slower decay rate. The percentage of tracer left in plasma after 60 minutes was significantly lower for native LDL than for methyl-LDL (71.4% vs 75.6%, mean difference (d) = 4.4%, sd 3.23, p < 0.01).

**Blood Contamination of Aortic Segments**

To test whether there was a significant amount of blood contamination in the aortic segments as prepared, we assessed this contamination using erythrocytes labeled with 51Cr. The labeled erythrocytes were injected into three rabbits, and perfusion was started 5 to 10 minutes after injection (mean 7.6 minutes).

The perfusion of the rabbits and dissection of the aortas was performed exactly as in the previous experiments. In samples obtained immediately before perfusion was started, the activity in the blood was 907,400 cpm/ml. Of this activity, 95% was present in the cells and only 5% in the plasma. From the radioactivity recovered in the aortic specimens, the amount of contaminating blood per gram of aortic tissue was calculated (Table 2). A total of only 383 nl of blood/g tissue was found to contaminate the tissue specimens. If a hematocrit of 40% is assumed, the LDL radioactivity due to contaminating plasma would lead to only 12% overestimation of the true flux rate.

**Linearity of Flux Rate during 60 Minutes**

To assess the linearity of flux rates over the 60 minutes after injection of the tracers (the interval used in most of these studies), the flux rates obtained in aortic segments perfused for the 60-minute experiments were compared with those in the 30-minute experiments. The observed entry rates were corrected for blood contamination estimated from 51Cr studies (see Table 2). The entry at 30 minutes was 54% of the entry at 60 minutes for native LDL.

**Table 2. Blood Contamination of Aortic Samples Determined with 51Cr-Labeled Erythrocytes**

<table>
<thead>
<tr>
<th>Aortic layers</th>
<th>Calculated blood contamination* (nl/g)</th>
<th>Corresponding plasma contamination (nl/g)</th>
<th>Error in LDL flux due to plasma contamination† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media-adventitia</td>
<td>234.5 ± 55.7</td>
<td>140.7</td>
<td>+ 12.3</td>
</tr>
<tr>
<td>Intima</td>
<td>148.6 ± 82.4</td>
<td>88.1</td>
<td>+ 12.5</td>
</tr>
<tr>
<td>Total</td>
<td>383.1 ± 121.3</td>
<td>228.8</td>
<td>+ 12.4</td>
</tr>
</tbody>
</table>

*Values are means ± SD from three rabbits. †Percentage of estimated total flux of native LDL.
and 55% for methyl-LDL. These data suggest that the flux rates were very nearly linear for 60 minutes.

Initial Entry Rate of Native LDL and Methyl-LDL into Cholesterol-Fed Rabbit Aortas

Three rabbits were fed ordinary chow supplemented with 2% cholesterol (wt/wt) for 3 days. The cholesterol feeding caused a five-fold increase in serum cholesterol level (250 vs 55 mg/dl). After the brief cholesterol feeding, the initial rates of entry of native LDL and methyl-LDL were determined as described. Three control rabbits were injected with the same tracers in parallel experiments. As shown in Figure 1, there was no significant difference between the aortic clearance of LDL in the cholesterol-fed rabbits (mean 1783 ± 696.1) and that in the control rabbits (mean 1513 ± 215.4 nl/g/hr) (Figure 1). The ratio of the uptake of methyl-LDL to that of native LDL was slightly, but not significantly, higher in the cholesterol-fed rabbits than in the controls (mean 1.163 ± 0.072 vs mean 1.073 ± 0.025, respectively).

As in the other normal rabbits, the three noncholesterol-fed controls showed a slower decay for methyl LDL than for native LDL, with a significantly higher fraction of injected methyl-LDL than of native LDL left in plasma after 60 minutes (mean difference, Δ = 3.9%, SD 1.10, p < 0.05). In the cholesterol-fed rabbits, no significant difference was observed, implying that the high concentration of cholesterol-rich lipoproteins in these rabbits effectively saturated and/or down-regulated LDL receptors.

Discussion

In the present studies, rates of influx were calculated from the total TCA-precipitable radioactivity found at 30 minutes and 60 minutes after injection of labeled LDL. This calculation implicitly assumes that only an acceptably small fraction of the labeled LDL entering the aortic wall over this time interval will have been lost from it. The fact that the calculated influx rate at 30 minutes was only slightly greater than that calculated from data obtained at 60 minutes tends to support these assumptions. If there had been a great deal of efflux or degradation, the apparent influx rate during the first 30 minutes would have exceeded that over the second 30 minutes and resulted in different calculated influx rates. As seen in Table 3, there was, in fact, a small difference (less than 10%) in the rates calculated from 30-minute and from 60-minute data, but the difference cannot be considered significant in view of the large variance.

Other investigators have noted a linear increase of tracer in the aorta for at least 1 hour after injection. This has been reported for LDL and albumin25 in the rabbit aorta and for phospholipids in the aorta of cockerels.26 In the present instance, we have data from a previous study12 on the actual rate of LDL degradation by the rabbit aorta in vivo. Those data were obtained using the "trapped ligand" technique in which a nondegradable ligand is covalently linked to the protein of interest and accumulates intracellularly after the lipoprotein (or other protein) has been degraded. The calculated steady-state rate of aortic degradation was found to be 0.000094 ± 0.000034 plasma LDL pools/g/day. From this value, we can estimate the maximum error using worst-case assumptions, namely: 1) that all the cells in the artery wall are exposed to labeled LDL at the plasma concentration; 2) that there is no time lag before the cells begin to generate degradation products. Making these worst-case assumptions we calculated that the maximum underestimate of influx would be about 450 nl/g/h against an observed influx of 1,697 nl/g/h (i.e., an error of about 25%). However, all of these assumptions are quite unrealistic. During the first 30 to 60 minutes after injection of tracer, there are marked concentration gradients across the thickness of the arterial wall.9 From cell culture studies it is well established that there is a significant lag period.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Aortic clearance of plasma LDL in three rabbits fed ordinary chow supplemented with 2% cholesterol for 3 days before the experiment and in simultaneously studied control, noncholesterol-fed rabbits.
before LDL enters cells and is transported to lysosomes and degradation products begin to exit.

A more realistic estimate of the magnitude of error introduced by degradation can be made when we recognize that the intima accounts for fully 40% of the total LDL degradation in the rabbit aorta and that the steep concentration gradient of LDL insures that the intima is exposed to a much higher concentration than the media. Consequently, a reasonable approximation can be made by using the data previously obtained for intimal degradation only. Further, we can extrapolate from cell culture studies and assume that there is a 15-minute delay between the exposure of cells to labeled LDL and the first appearance of degradation products. Using these assumptions we calculated a minimum error of 140 nl/g/hr, which is 8.2% of the mean of the measured values for LDL transport at 60 minutes (Table 3).

Our previous studies showed that the rate of degradation of reductively methylated LDL in the rabbit aorta is about 50% that of unmodified LDL. Using this factor and the appropriate exponential decay rates for reductively methylated LDL, we can similarly estimate a minimal error for methyl LDL; that turns out to be 72 nl/g/hr or 4.1% of the measured transport rate at 60 minutes (Table 3). The results of these calculations are summarized in Table 4, and the mathematical expressions used are indicated there. It is interesting to note that after using the minimum correction figures, transport values for native and methyl LDL are remarkably close to one another. This further reinforces the main result of this paper, namely, that transport of LDL into the artery wall does not depend to any large extent on interaction with LDL receptors.

Listed in Table 4 are the steady-state LDL degradation rates from our previous study expressed in the same units as the fluxes measured in the present studies for ease of comparison. In that earlier study, estimates of total native LDL flux into the aorta were made in a limited number of rabbits. It was calculated that the intima degraded 11.5% of the LDL flux entering the aorta; the remainder of the aorta (media plus adventitia) degraded 17.1% of the total LDL influx.

Table 4. Comparison of LDL Flux and LDL Degradation In Normal Rabbit Aorta

<table>
<thead>
<tr>
<th></th>
<th>LDL flux into aorta and estimated errors due to degradation (nl/g/hr)</th>
<th>Total flux of LDL corrected for LDL degradation (nl/g/hr)</th>
<th>LDL degradation rates at steady state (nl/g/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start of perfusion</td>
<td>Measured fluxes of LDL*</td>
<td>Calculated correction due to degradation</td>
</tr>
<tr>
<td>Native</td>
<td>30 min</td>
<td>1844</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>1697</td>
<td>450</td>
</tr>
<tr>
<td>Methyl</td>
<td>30 min</td>
<td>1952</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>1772</td>
<td>231</td>
</tr>
</tbody>
</table>

*Values are means taken from Table 3. ND = not determined.
† It is assumed that all aortic cells were exposed to full plasma concentration of labeled LDL right from zero time and that there was no lag time in generation of degradation products. Let $k_a$ represent the total aortic wall degradation rate measured previously by Carew et al. (reference 12) and let $X(t)$ represent the plasma concentration of labeled LDL at time $t$. Then degradation of labeled LDL becomes:

$$\int_0^{k_a} X(t) \, dt$$

Over the first hour, $X(t)$ is well approximated as a single exponential decay. The numerical result of integration is divided by the injected dose and multiplied by plasma volume to yield an equivalent volume of plasma "cleared" by aortic degradation.

‡ It is assumed that degradation over short times occurred principally in the intima at a rate $k_j$ taken from Carew et al. (reference 12), and that there was a 15-minute lag before degradation products were formed and that degradation thereafter could be related to $^{125}$I-LDL concentration in plasma 15 minutes earlier. Then degradation of $^{125}$I-LDL becomes:

$$\int_0^{15} k_j X(t) \, dt$$

where $T$ is either 0.5 or 1 hour. If $t = t' + 0.25$ is substituted for the dummy variable of integration $t$ and if it is noted that $X(t'<0) = 0$, the equivalent expression for degradation is:

$$\int_0^{T-0.25} k_j X(t') \, dt'$$

Evaluation of this integral and conversion to "clearance" units yields the minimal error due to degradation.

§Calculated from data in Table 1 of Carew et al. (reference 12) for a 3 kg rabbit having a plasma volume of 135 ml.
‖ Calculated from data in Table 1 and Figure 8 (48-hour studies) from Carew et al. (reference 12).
Because LDL does not accumulate in the normal rabbit aorta at steady state, it was concluded that most of the LDL flux into aorta (i.e., 71.4%) must be bidirectional. A comparison of the calculated fluxes from the present series of studies with the previous estimates of LDL degradation rates yields the same conclusion. If we compare intimal and whole aorta degradation rates measured at the total LDL flux (60-minute studies calculated with "minimal correction", 1837 nL/g/hr), we find that 11.6% of LDL flux is degraded by the intima and 28.7%, by the whole aorta including intima. This leaves 71.3% of the flux which must be bidirectional. These values are remarkably close to the previous estimates.

Because the intima degrades more native LDL than methylated LDL (Table 4, Column 6), the question arises as to why this is not reflected in a greater measured flux of native vs methyl LDL. The difference between absolute intimal degradation rates of native and methyl LDLs is only 102 nL/g/hr, (i.e., only about 5% of total flux) and is well within the variance of the flux measurements (Table 3).

A source of error that could obscure differences in flux rates is the blood contamination of the arterial specimens. The studies performed here with 51Cr-labeled erythrocytes, however, show that with the techniques used, blood contamination could not affect the results in any major way. The fact that there was some 51Cr in the plasma suggests that at least some of the 51Cr in the tissue represented diffusion of free chromium into the artery rather than erythrocyte contamination. Thus, the calculated 12% plasma contamination (Table 2) probably overestimates the true degree of contamination.

To further test the role of the receptor in controlling the LDL flux into rabbit aorta, the cellular receptor content was down-regulated by short-term cholesterol feeding before the experiments were performed. The cholesterol feeding did not significantly change the aortic LDL transport. It has been suggested that the high flux rate of LDL found by others after long-term cholesterol feeding was caused by a change in endothelial integrity. It could be argued that in the present study a down-regulation of receptor-mediated uptake was exactly opposed by an equal increase in nonspecific uptake (induced, for example, by endothelial damage due to the cholesterol feeding), but this seems unlikely.

The data of the present study suggest that the bulk of uptake of LDL into the normal rabbit aorta is not receptor-dependent. It has recently been shown that 40% to 50% of the LDL degraded in the normal rabbit aortic intima is degraded via the receptor pathway. Thus, while the receptor makes an important contribution to degradation of LDL in the aortic wall, the total flux of LDL into the arterial tissue is mostly receptor-independent. A similar conclusion has been reached by Vasile et al based on electron microscopic studies of the endothelial uptake of LDL by perfused rat arteries. This conclusion is supported by other observations that the major factors determining flux rates of LDL and other macromolecules into aorta are their molecular size and concentration gradients and endothelial integrity.

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