Influx In Vivo of Low Density, Intermediate Density, and Very Low Density Lipoproteins Into Aortic Intimas of Genetically Hyperlipidemic Rabbits

Roles of Plasma Concentration, Extent of Aortic Lesion, and Lipoprotein Particle Size as Determinants

Børge G. Nordestgaard, Anne Tybjaerg-Hansen, and Barry Lewis

To compare the atherogenic potential of low density lipoprotein (LDL), intermediate density lipoprotein (IDL), and very low density lipoprotein (VLDL) under conditions where plasma levels of these lipoproteins are elevated, the influx of cholesterol in these lipoproteins into the aortic intima was measured in vivo in genetically hyperlipidemic rabbits from the St. Thomas’s Hospital strain, an animal model that shares many of the features of the human disorder familial combined hyperlipidemia. Univariate linear regression showed that the arterial influx of LDL cholesterol (n = 25), IDL cholesterol (n = 14), and VLDL cholesterol (n = 10) was positively and linearly associated with plasma concentrations of LDL cholesterol in the range 0.2–6.4 mmol/l, of IDL cholesterol in the range 0.1–7.0 mmol/l, and of VLDL cholesterol in the range 0.7–8.5 mmol/l, respectively, and also with the extent of lesions in the arterial intima in the range 0–100% of the surface area. Multiple linear regression suggested that the arterial influx of LDL, IDL, and VLDL cholesterol was linearly dependent on plasma concentration, independent of lesion size. Furthermore, it appeared that the arterial influx of the three lipoproteins was linearly dependent on the extent of the lesions, independent of lipoprotein concentration. When influx was normalized for plasma concentration (intimal clearance) and for lesion size (compared within the same aorta), the intimal clearance of the larger IDL and VLDL particles was 15–35% less than that of the smaller LDL particles. These findings suggest that the quantitatively most important mechanism for transfer of plasma lipoproteins into the arterial intima involves nonspecific molecular sieving and that at elevated plasma levels, IDL and VLDL share with LDL the potential for causing atherosclerosis. (Arteriosclerosis and Thrombosis 1992;12:6–18)

The human disorder familial combined hyperlipidemia is associated with premature atherosclerotic heart disease; it is the most common form of familial hyperlipidemia in survivors of myocardial infarction. Affected patients exhibit elevated plasma levels of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and/or low density lipoprotein (LDL), most likely caused by an overproduction of these lipoproteins. The atherogenic role of LDL is well established, and there is also evidence that IDL may be atherogenic, but at present, little is known about the relative atherogenicity of these three different apo-lipoprotein (apo) B-containing lipoproteins.

A recently described strain of genetically hyperlipidemic rabbits, the St. Thomas’s Hospital rabbit strain, has many features in common with the human disorder familial combined hyperlipidemia. These rabbits have elevated plasma levels of VLDL, IDL, and/or LDL, which seem to be caused by overproduction of the lipoproteins. The hyperlipidemic rabbits, when fed ordinary rabbit chow, develop arterial lesions resembling human atherosclerosis.
TABLE 1. Description of Arterial Wall Kinetic Studies in Rabbits of the St. Thomas's Hospital Strain

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Validation experiment</th>
<th>Influx experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Contamination (using LDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>2-5</td>
<td>Contamination (using IDL and VLDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>6</td>
<td>Equivalency of $^{125}$I and $^{131}$I (using LDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>7</td>
<td>“Sink” assumption (using LDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>8-12</td>
<td>Labeling of arterial lipoproteins (using LDL, IDL, and VLDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>13</td>
<td>$37^\circ$C incubation (using LDL)</td>
<td>LDL</td>
</tr>
<tr>
<td>14-16</td>
<td>...</td>
<td>LDL</td>
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<td>17-19</td>
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<td>LDL</td>
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<td>20-22</td>
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<td>LDL</td>
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<tr>
<td>23-25</td>
<td>...</td>
<td>LDL</td>
</tr>
<tr>
<td>26-37</td>
<td>...</td>
<td>LDL</td>
</tr>
<tr>
<td>38-45</td>
<td>...</td>
<td>LDL</td>
</tr>
<tr>
<td>46-49</td>
<td>...</td>
<td>LDL</td>
</tr>
</tbody>
</table>

For methodology, see “Protocol for Influx Experiments” and “Validation of Conditions for Influx Experiments” in the “Methods” section. Several of the rabbits (26–49) were also studied for other purposes, which will be reported elsewhere.

LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; Sf, flotation rate in Svedberg units.

imal model, therefore, offers the opportunity to study the relative atherogenicity of VLDL, IDL, and LDL under conditions where plasma levels of these lipoprotein fractions are elevated.

We have made the assumption that the atherogenic potential of an apo B-containing lipoprotein reflects the rate at which it may deliver cholesterol into the arterial intima; on this basis we have compared the arterial influx in vivo of VLDL, IDL, and LDL in this rabbit strain. Furthermore, we have attempted to delineate some determinants of influx.

Methods

Animals

The 49 rabbits (Table 1), 32 females and 17 males, weighing 3.3–5.6 kg and aged 9–35 months, were descendants of the founder male of the St. Thomas's Hospital rabbit strain; the parents of the rabbits were either two hyperlipidemic rabbits or one hyperlipidemic and one normolipidemic rabbit, the latter also being a New Zealand White rabbit. SG 1 pellets (Grain Harvesters Ltd., Wigham, UK) were fed to the rabbits ad libitum. Plasma cholesterol and triglyceride levels in the 49 rabbits ranged from 1.2 to 23.1 mmol/l and from 0.5 to 9.1 mmol/l, respectively. Experimental protocols were in accordance with the UK Home Office guidelines for experiments with animals.

Preparation of Labeled Lipoproteins

VLDL, IDL, LDL, and Sf 12–60 lipoprotein (where Sf is the rate of flotation in Svedberg units) were prepared from freshly drawn, nonfasting rabbit blood containing the anticoagulant Na$_2$EDTA (1.2 mg/ml, Sigma Chemical Co., Poole, UK); the antibiotics chloramphenicol (80 μg/ml; Sigma) and gentamicin sulfate (80 μg/ml; Sigma); and the protease inhibitors e-amino-n-caproic acid (2.6 mg/ml; Sigma), benzamidine (10 μg/ml; Sigma), and aprotenin (5–9 kallikrein units/ml; Sigma). Lipoproteins were separated promptly at 4°C from an average of 12 ml plasma with salt solutions and with the previously mentioned concentrations of the two antibiotics, the three protease inhibitors, and 0.1 mg/ml Na$_2$EDTA. VLDL ($d<1.006$ g/ml), IDL ($1.006$ g/ml<$d<1.019$ g/ml), and LDL ($1.019$ g/ml<$d<1.063$ g/ml) were isolated as the top fractions after sequential ultracentrifugation in a Kontron TFT 45.6 rotor (Watford, Hertfordshire, UK), for $2.4\times10^6$ $g$·min·hour (where $av=average$) in solvent densities of $1.006$, $1.019$, and $1.063$ g/ml, respectively. To isolate Sf 12–60 lipoprotein, Sf 60 lipoprotein was first isolated and discarded; in each tube, 3 ml plasma was overlaid with 2 ml of $d=1.006$ g/ml salt solution in $13\times64$-mm polyallomer tubes (Kontron) and ultracentrifuged for $1.1\times10^6$ $g$·min$^{-1}$ in a Kontron TFT 45.6 rotor; the top 1.5 ml was the Sf 60 lipoprotein. Subsequently, Sf 12–60 lipoprotein was isolated as the top fraction after ultracentrifugation at $d=1.019$ g/ml. Lipoprotein fractions were washed by a further ultracentrifugation step at their upper density limits to minimize contamination.

Before iodination, lipoproteins were equilibrated with saline containing antibiotics, protease inhibitors, and Na$_2$EDTA as described (“saline”) with the use of PD-10 gel filtration columns (Sephadex G-25M, Pharmacia, Uppsala, Sweden). Iodination was performed according to the modification by Bilheimer et al of McFarlane's iodine monochloride method as follows. In 0.4–1.8 ml saline containing lipoproteins, pH was adjusted to 10 with 0.3–0.5 ml glycine buffer
The volume of distribution was calculated as the injected dose (counts per minute) divided by radioactivity concentration in plasma (counts per minute per millimeter) 10 minutes after injection and divided by the weight of the rabbit (kilograms). Because these values of distribution are not larger than plasma volumes of cholesterol-fed rabbits as determined with albumin labeled in vivo, it is unlikely that even 1–2% of the dose was denatured; such a damaged dose would be removed rapidly by the liver, and the volume of distribution would then have been larger than plasma volume.

Nonfasting blood samples containing antibiotics, protease inhibitors, and Na₂EDTA as described were drawn from ear veins at timed intervals after injection of the dose, and the rabbits were killed 3.3±0.1 hours (n=40) later by intravenous injection of pentobarbital sodium (50–100 mg/kg; May & Baker Ltd., Dagenham, UK). The thorax was opened and the rabbit was perfused with 1 l saline introduced into the left ventricle of the heart after the inferior vena cava was severed. Subsequently, the aorta was excised, the adventitia was removed, and the vessel was opened longitudinally and rinsed with saline. The aorta was fixed with pins on a corkboard, and the area was outlined on graph paper; the vessel was divided into the aortic arch (from the heart to the first intercostal arteries), the thoracic aorta (to the diaphragm), and the abdominal aorta (to the bifurcation). The area of the arch, thoracic aorta, and abdominal aorta was 6.2±0.1 cm², 7.7±0.1 cm², and 7.7±0.1 cm² (n=49), respectively. As a measure of the extent of atherosclerosis, the fraction of the total aortic intimal surface in each of these aortic regions that showed lesions was graded visually as 0%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. The aortas were all graded by the same person (B.G.N.) without knowledge of the rabbits' lipoprotein levels or kinetic characteristics. To validate grading of aortic lesions, the aortas, thoracic aortas, and abdominal aortas from another set of rabbits from the St. Thomas’s Hospital strain were graded visually by B.G.N. and compared with the percentage of surface area showing lesions as determined independently by use of a Joyce-Loebl planimeter after staining with oil red O. The linear regression of one grading method versus the other had an R² of 0.94 (p<0.001, 78 samples), and the line had an intercept not significantly different from zero.

The intima–inner media was stripped from the remaining media in each of the three aortic regions, minced with scissors, and placed in a shaker with 1.5 ml saline for 3 days at 4°C to extract lipoproteins (see “Validation” section). This suspension then underwent isopropanol precipitation (see below).

The VLDL, IDL, LDL, and HDL (d>1.063 g/ml) were isolated from plasma samples by sequential ultracentrifugation. In experiments with Sf 12–60 lipoprotein (rabbits 23–25), Sf>60 lipoprotein, Sf 12–60 lipoprotein, LDL, and HDL were separated. Enzymatic methods were used to measure cholesterol (CHOD-PAP, Boehringer-Mannheim, Mannheim, FRG) and triglyceride (GPO-PAP, Wako Chemicals GmbH, Neuss, FRG) in plasma and lipoprotein fractions. Recovery of cholesterol for

Protocol for Influx Experiments

Because plasma concentrations of IDL and VLDL after an overnight fast may not represent the average lipoprotein levels to which the arterial wall is exposed, influx experiments were performed with nonfasted animals that were fed ordinary chow ad libitum. Of the 40 rabbits used for influx experiments (rabbits 1, 6, 7, and 13–49; Table 1), 23 were injected with labeled lipoproteins, and the remaining 17 were injected with labeled lipoproteins similar to their own lipoproteins; all labeled lipoproteins were from rabbits of the St. Thomas’s Hospital strain. The labeled preparations (5.2±0.5 ml, n=40) containing either two labeled lipoprotein fractions (rabbits 14–25) or only one fraction (rabbits 1, 6, 7, 13, and 26–49) labeled with 125I (177±23 μCi, n=30) and/or 131I (34±4 μCi, n=22) were injected intravenously. Volumes of distribution of these injected doses were 30.2±1.0 ml/kg and 30.6±1.0 ml/kg for 125I- and 131I-labeled lipoproteins, respectively; the volume of distribution was calculated as the injected dose (counts per minute) divided by radioactivity concentration in plasma (counts per minute per millimeter) 10 minutes after injection and divided by the weight of the rabbit (kilograms). Because these values of distribution are not larger than plasma volumes of cholesterol-fed rabbits as determined with albumin labeled in vivo, it is unlikely that even 1–2% of the dose was denatured; such a damaged dose would be removed rapidly by the liver, and the volume of distribution would then have been larger than plasma volume.

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the three consecutive ultracentrifugations together was 82±0.7% (80 samples). Reported values for plasma and lipoprotein lipids (corrected for loss during ultracentrifugation) are mean values from two separate ultracentrifugal fractionations of samples from each animal.

**Analyses**

Apo B was precipitated with isopropanol in aliquots of the doses, in minced and saline-treated arterial extracts, and in aliquots of lipoprotein fractions from plasma samples; human apo B–containing lipoproteins (d<1.063 g/ml) were added to all samples as carriers before precipitation. A 1- or 1.5-ml sample was combined with 1 or 1.5 ml 100% isopropanol (BDH Ltd., Poole, UK) in a 15×56-mm plastic counting vial (Sterilin Ltd., Feltham, UK). After 1 minute of vigorous mixing, the samples were incubated for 3 days at 4°C and then centrifuged at 1,500g for 30 minutes at 4°C. The supernatant was removed, and the precipitate (for arterial tissue, this included the minced tissue) was washed twice with 2 ml 50% isopropanol and finally with 2 ml 100% isopropanol to ensure complete lipid extraction. To obtain equal counting conditions, 2 ml 50% isopropanol was added to the precipitate before it and the various washes were counted in an LKB-Wallac 1280 Ultragamma double-channel gamma counter (Pharmacia-LKB Biotechnology, Milton Keynes, UK). Part of the radioactivity in the washes was assumed to be due to free iodine; hence, trichloroacetic acid (TCA) precipitation was performed on other aliquots on the same day as the isopropanol precipitation, and the isopropanol-precipitable counts were corrected accordingly; the counts in the TCA supernatant (free iodine counts) were subtracted from the counts in the isopropanol washes before calculation of the percent of label precipitable with isopropanol, as shown in Table 2. For arterial tissue, where all tissue was used for isopropanol precipitation, the medial tissue was used to estimate TCA-precipitable counts. On the same day, aliquots of the dose and of plasma lipoproteins, all with added carrier plasma, were also extracted and precipitated with chloroform/methanol (1:1, vol/vol) to estimate extractable lipid counts. As with isopropanol precipitation, extractable lipid counts were corrected for counts due to free iodine, which were estimated as counts in the supernatant after TCA precipitation.

**Validation of Conditions for Influx Experiments**

To estimate the contribution of adhering plasma to radioactivity in arterial intima–inner media, 125I- and/or 131I-labeled lipoproteins were injected intravenously into rabbits 1–5 (Table 1) 10–13 minutes before removal of arterial tissue, as described above. Radioactivity present in the intima–inner media was assumed to represent surface contamination. Contamination was similar whether labeled LDL (one sample), IDL (three samples), or labeled VLDL (two samples) was used, and it was similar in the intima with and without lesions. The average contamination values for the arch, thoracic aorta, and abdominal aorta were 7.6±2.5 nl/cm² (six samples), 29±6 nl/cm², and 8.8±2.0 nl/cm². These mean values are similar to values obtained previously in three cholesterol-fed rabbits that had been injected with iodinated LDL. Influx and intimal clearance were, in the present study of all rabbits, calculated based on uncorrected radioactivity values for intima–inner media as well as on radioactivity in the intima–inner media corrected for contamination. After correction for plasma contamination, radioactivity in the intima–inner media of the 40 rabbits used for influx studies was 81±2% (156 samples) of the uncorrected values. Contamination-corrected values are reported in the present article; our conclusions were similar whether

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**Table 2.** Distribution of Iodine Label in Injected, Labeled Lipoproteins (Dose), Plasma Lipoproteins Obtained 1 Hour After Injections of Dose, and in Intima–Inner Media of the Aortic Arch From Rabbits of the St. Thomas’ Hospital Strain

<table>
<thead>
<tr>
<th>Labeled lipoprotein</th>
<th>LDL (8)</th>
<th>IDL (5)</th>
<th>VLDL (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected labeled lipoprotein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B</td>
<td>82±2</td>
<td>91±2</td>
<td>62±2</td>
</tr>
<tr>
<td>Lipid</td>
<td>12±2</td>
<td>6±1</td>
<td>29±6</td>
</tr>
<tr>
<td>Other apos</td>
<td>7±1</td>
<td>4±1</td>
<td>10±3</td>
</tr>
<tr>
<td>Plasma lipoprotein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B</td>
<td>88±2</td>
<td>91±1</td>
<td>64±7</td>
</tr>
<tr>
<td>Lipid</td>
<td>7±1</td>
<td>4±1</td>
<td>27±6</td>
</tr>
<tr>
<td>Other apos</td>
<td>5±1</td>
<td>4±2</td>
<td>9±1</td>
</tr>
<tr>
<td>Aortic arch</td>
<td>71±6</td>
<td>75±5</td>
<td>53±8</td>
</tr>
</tbody>
</table>

Values are percent and are mean±SEM. Percent label in apolipoprotein (apo) B was estimated as isopropanol-precipitable counts, adjusted for label due to free iodine. Percent label in lipid was estimated as chloroform/methanol-extractable counts, adjusted for label due to free iodine. Percent label in other apolipoproteins (apos) was calculated as 100% minus apo B and lipid counts. Numbers in parentheses indicate number of samples. LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein.
validate this, 125I-labeled LDL and 131I-labeled LDL were injected intravenously into rabbit 7 at two fed rabbits and iodinated LDL. 19

Excised an average 3.6 hours later, minced, and saline injected intravenously into rabbit 6. The arterial influx of LDL cholesterol (picomoles per nanoliter) was 95%, 101%, and 105% of the influx for 125I-labeled LDL in the arch, thoracic aorta, and abdominal aorta, respectively. Similar, almost identical, behavior of 125I- and 131I-labeled lipoproteins for arterial influx measurements has previously been observed in cholesterol-fed rabbits. 20

Arteries were exposed to circulating labeled lipoproteins for an average of only 3.3 hours, with the intention that transfer of labeled lipoproteins from the arterial intima during the exposure period would be small compared with arterial influx; that is, that the arterial intima would behave as a "sink." To validate this, 125I-labeled LDL and 131I-labeled LDL were injected intravenously into rabbit 7 at two different time points before removal of the aorta (Figure 1). Arterial influx of LDL cholesterol increased almost linearly with time for 5 hours, which suggests that for such a short influx period, the sink assumption is appropriate. If eflux of labeled LDL had been significant compared with influx of labeled LDL during the 5 hours, then the three curves in Figure 1 would have been curvilinear with a concavity toward the x axis. Results similar to those in Figure 1 have previously been reported for two cholesterol-fed rabbits and iodinated LDL. 19

After intravenous injection of iodinated LDL, IDL, and/or VLDL into rabbits 8–12, the aorta was excised an average 3.6 hours later, minced, and saline washed twice with saline. The combined washes with added carrier plasma were ultracentrifuged to isolate arterial VLDL, IDL, and LDL. These fractions were precipitated with isopropanol, and the counts in the precipitated apo B were determined.

To test if the incubation of the labeled lipoproteins at 37°C for 48 hours with HDL and plasma lipid transfer protein did affect arterial influx or intimal clearance of such lipoproteins, one aliquot of LDL was iodinated with 125I and incubated as described. Another aliquot of the same LDL kept at 4°C was iodinated with 131I 3 days later, when the 125I-labeled LDL had been reisolated. The two aliquots were mixed, dialyzed overnight against saline, and then injected intravenously into rabbit 13. Arterial influx of the 37°C-incubated LDL was 89%, 93%, and 91% of that of nonincubated LDL in the arch, thoracic aorta, and abdominal aorta, respectively. Considering the large variation usually found in arterial influx measurements, the two sets of influxes are similar. Furthermore, the plasma decay curves for the incubated and nonincubated LDL never differed by more than 1%.

Calculations

Calculations of arterial intimal clearance and influx of lipoproteins were based on radioactivity in apo B in plasma lipoproteins and in the total arterial intima–inner media after correction for contamination; in 10 rabbits used for measurements of LDL influx only, influx calculations were based on total counts in plasma and in arterial intima–inner media (corrected for contamination) as previously performed. 19, 20 The 125I and 131I counts (above background and after spill-over correction in the intima–inner media) used for influx calculations were 711 ± 99 cpm (90 samples) and 130 ± 23 cpm (66 samples); backgrounds for the 125I and the 131I channels were 17–25 cpm and 38–45 cpm, respectively.

Arterial intimal clearance of lipoproteins (nanoliters per square centimeter per hour) was calculated by the sink method; radioactivity in the intima–inner media (counts per minute per square centimeter) was divided by the time-averaged radioactivity of all plasma lipoprotein fractions combined during the average 3.3-hour influx period (counts per minute per nanoliter) and by the length of the influx period (hours). Arterial influx of lipoprotein cholesterol (picomoles per square centimeter per hour) was calculated as intimal clearance (nanoliters per square centimeter per hour) multiplied by the plasma concentration of the appropriate lipoprotein cholesterol (picomoles per nanoliter). The calculation of lipoprotein cholesterol influx assumes that the intimal clearance measured for apo B is also valid for the cholesterol moiety of the lipoproteins. Stender and Zilversmit 21 have provided evidence that in cholesterol-fed rabbits, cholesteryl ester and protein of

**Figure 1.** Line plot showing validation of the "sink" assumption for labeled low density lipoprotein (LDL) in three aortic sites in rabbit 7. Iodine-131–labeled LDL was injected intravenously at time zero, and 2 hours and 20 minutes later, the same LDL labeled with 125I was injected. Influx was calculated as radioactivity in tissue (counts per minute per square centimeter), corrected for contamination, divided by the time-averaged radioactivity concentration in plasma (counts per minute per nanoliter), and multiplied by plasma LDL cholesterol (picomoles per nanoliter).
TABLE 3. Distribution of Apolipoprotein B Iodine Label Among Plasma Lipoproteins Obtained at the End of the Influx Period

<table>
<thead>
<tr>
<th>Labeled lipoprotein</th>
<th>LDL (15)</th>
<th>IDL (14)</th>
<th>VLDL (10)</th>
<th>SF 12–60 (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF &gt;60</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>4±1</td>
</tr>
<tr>
<td>SF 12–60</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>90±1</td>
</tr>
<tr>
<td>VLDL</td>
<td>0±0</td>
<td>3±2</td>
<td>87±4</td>
<td>...</td>
</tr>
<tr>
<td>IDL</td>
<td>3±1</td>
<td>72±6</td>
<td>12±3</td>
<td>...</td>
</tr>
<tr>
<td>LDL</td>
<td>96±1</td>
<td>24±6</td>
<td>1±0</td>
<td>6±1</td>
</tr>
<tr>
<td>HDL</td>
<td>1±0</td>
<td>1±1</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

Values are percent and are mean±SEM. Plasma was separated by ultracentrifugation, the various lipoprotein fractions were precipitated with isopropanol, and finally the radioactivity in the precipitated apolipoprotein (apo) B was determined. Numbers in parentheses indicate number of samples.

LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; SF, flotation rate in Svedberg units; HDL, high density lipoprotein.

LDL enter the arterial intima-inner media in the same proportions as are present in plasma LDL.

Statistics

Values are presented as mean±SEM. Univariate and multiple linear-regression analyses were performed with the MINITAB program. The Wilcoxon matched-pairs signed-rank test was performed manually.

Electron Microscopy of Negatively Stained Lipoproteins

The average diameters of LDL, IDL, and VLDL particles from two rabbits of the St. Thomas’s Hospital strain were determined from electron photomicrographs of negatively stained lipoproteins. In brief, lipoprotein fractions were dialyzed against a volatile buffer consisting of 125 mmol/l ammonium acetate, 2.6 mmol/l ammonium carbonate, and 0.26 mmol/l Na₂EDTA (pH 7.4) and then mixed with the negative stain phosphotungstate just before examination and photography in a transmission electron microscope at a magnification of ×29,000 on the negative. The diameters of 100 randomly selected lipoprotein particles were determined from prints at a total magnification of ×200,000. To avoid inclusion of artificially large VLDL diameters due to flattening of lipoprotein particles, two different approaches were used. Some aliquots of VLDL were fixed with a 2% solution of OsO₄ in 150 mmol/l sodium cacodylate buffer (pH 7.4) before negative staining, whereas other aliquots were kept at 37°C during the entire negative staining procedure.

Results

Labeled Lipoproteins in Plasma and Arterial Intima

The major part of the iodine label was attached to the apo B of LDL, IDL, and VLDL, whether measured in the dose, in plasma lipoproteins, or in the intima-media of the aortic arch (Table 2). The present article is concerned only with the label in apo B in plasma and intima-media.

When labeled LDL was injected intravenously, >95% of the apo B label in plasma remained in the LDL fraction throughout the influx period (Table 3). However, when labeled IDL was injected, about 25% of the apo B label appeared in plasma LDL. Similarly, after intravenous injection of labeled VLDL, about 10% of the apo B label appeared in plasma LDL, and after injection of labeled SF 12–60 lipoprotein, about 10% of the apo B label appeared in apo B of LDL.

In experiments with labeled LDL, about 95% of the apo B label in arterial VLDL, IDL, and LDL

TABLE 4. Distribution of Apolipoprotein B Iodine Label Among Lipoproteins Isolated From Arterial Intima-Inner Media of Rabbits 8–12

<table>
<thead>
<tr>
<th>Labeled lipoprotein</th>
<th>LDL (4)</th>
<th>IDL (6)</th>
<th>VLDL (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial VLDL</td>
<td>3±3</td>
<td>3±2</td>
<td>34</td>
</tr>
<tr>
<td>Arterial IDL</td>
<td>4±3</td>
<td>42±8</td>
<td>40</td>
</tr>
<tr>
<td>Arterial LDL</td>
<td>94±4</td>
<td>55±10</td>
<td>27</td>
</tr>
</tbody>
</table>

Values are percent and are mean±SEM, based on the number of tissues shown in parentheses. The VLDL fraction was assayed in two segments of aorta (arch and combined thoracic and abdominal aortas) from one rabbit, labeled LDL was assayed in the same two segments of aorta in three different rabbits, and labeled LDL was assayed in two aortic segments in one rabbit and in the whole aorta combined in two other rabbits. Some rabbits had two different labeled lipoprotein fractions injected simultaneously. After a 3–4-hour exposure to intravenously injected labeled lipoproteins, arterial intima-inner media samples were minced with a pair of scissors and then extracted in saline at 4°C for 24–72 hours. Subsequently, lipoproteins were separated in the saline extracts by ultracentrifugation and finally, the lipoprotein fractions were subjected to apolipoprotein B precipitation with isopropanol.

LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein.
FIGURE 2. Plots of influx of low density lipoprotein (LDL) cholesterol into various aortic intima-inner media in 25 rabbits, as a function of the extent of aortic lesions and of plasma LDL cholesterol. Influx was calculated as radioactivity in tissue (counts per minute per square centimeter), corrected for contamination, divided by the time-averaged radioactivity concentration in plasma (counts per minute per nanoliter) and by the length of the influx period (hours), and then multiplied by plasma LDL cholesterol (picomoles per nanoliter). Note that the y axis (influx) of thoracic and abdominal aortas is half that of the aortic arch.

Arterial Influx of Low Density Lipoprotein, Intermediate Density Lipoprotein, and Very Low Density Lipoprotein Cholesterol

Previously, it has been difficult to demonstrate that arterial influx of lipoproteins is dependent on their plasma concentration. One possible reason is a large interindividual variation in influx (despite similar plasma concentrations), probably caused by differences in the extent and stage of arterial lesions. In the present article, these potential determinants have been separately assessed as shown in Figures 2–4, so that arterial influx can be independently assessed as a function of lipoprotein cholesterol concentration in plasma and as a function of the extent of lesions in the arterial intima.

Arterial influx of LDL cholesterol increased linearly with increasing extent of arterial lesions and also with increasing plasma LDL cholesterol (Table 5). Arterial influx of VLDL cholesterol also increased linearly, both with the extent of arterial lesions and with increasing VLDL cholesterol in plasma (Table 5). Plasma LDL cholesterol and plasma IDL cholesterol were both positively and linearly correlated with percentage of the intima with lesions at all three aortic sites (p<0.05), but for plasma VLDL cholesterol, this was only true for the aortic arch (data not shown).

By using multiple linear regression of lipoprotein cholesterol and the percent area showing lesions simultaneously as predictors of influx, the independent effect of each predictor was evaluated (Table 6). Concentrations of LDL cholesterol in plasma and percent lesions in the intima were each positively and linearly associated with arterial influx of LDL cholesterol, although for plasma LDL cholesterol, this was significant at the 0.05 level only for the aortic arch. When the arterial influx of LDL cholesterol value was square-root transformed, a better fit was obtained for the multiple linear regression (R² increased from 0.65, 0.64, and 0.73 to 0.82, 0.81, and 0.84 for the arch, thoracic aorta, and abdominal aorta, respectively); LDL cholesterol concentration was then positively associated with arterial influx at all three aortic sites. Plasma IDL cholesterol was independently linearly positively associated with influx of IDL cholesterol in two of three aortic sites, but in these IDL experiments, the percentage of arterial surface showing lesions only reached a statistically significant, independent linear association with influx at one aortic site. Plasma VLDL cholesterol was...
Nordestgaard et al Lipoprotein Influx Into Arterial Intima 13

AORTIC ARCH  THORACIC AORTA  ABDOMINAL AORTA

FIGURE 3. Plots of influx of intermediate density lipoprotein (IDL) cholesterol into various aortic intima-inner media in 14 rabbits, as a function of the extent of aortic lesions and of plasma IDL cholesterol. Note that the y axis (influx) of thoracic and abdominal aortas is half that of the aortic arch.

Terol was independently linearly positively associated with influx at all three aortic sites, and similarly, lesion percentage was linearly positively associated with influx in two of the three tissues.

The univariate and multiple linear regressions in Tables 5 and 6 were also performed with lipoprotein concentration and lesion percentage as predictors of the square-root-transformed or logarithmically transformed lipoprotein influx values. Except for the multiple linear regression in the LDL experiment (see above), neither the logarithmically nor the square-root-transformed models were superior to the simple linear fit (data not shown).

Arterial Intimal Clearance of Low Density Lipoprotein, Intermediate Density Lipoprotein, and Very Low Density Lipoprotein

Because arterial influx of LDL, IDL, and VLDL cholesterol appears to be linearly dependent on their respective plasma concentrations, it is reason-
able to express arterial uptake of lipoproteins as an intimal clearance, that is, as arterial influx normalized for differences in plasma concentration (Figure 5). For the animals shown in Figure 5, because intimal clearances of two different lipoprotein fractions were compared within each aorta (data points connected with a line), differences in arterial lesion size between aortas did not affect the comparison. Intimal clearance of IDL, Sf 12–60 lipoprotein, and VLDL was 71 ±4% (nine samples), 67 ±8% (nine samples), and 85 ±7% (nine samples), respectively, of the simultaneously measured LDL intimal clearance. VLDL intimal clearance was 101 ±9% (nine samples) of the simultaneously measured IDL intimal clearance. Using the Wilcoxon matched-pairs signed-rank test on intimal clearances for all 12 rabbits, we tested whether lipoprotein size did influence intimal clearance; intimal clearance for the large lipoprotein in the pair was lower than that for the small lipoprotein in the arch (p<0.05), thoracic aorta (p<0.02), and abdominal aorta (p<0.02). Similar results were obtained with logarithmically transformed intimal clearances.

Size of Low Density Lipoprotein, Intermediate Density Lipoprotein, and Very Low Density Lipoprotein

The diameters of negatively stained lipoproteins (Figure 6) in the two rabbits examined were 27.0 ±0.3 nm (100 samples) and 27.4 ±0.3 nm for LDL, 35.5 ±0.6 and 34.7 ±0.3 nm for IDL, 45.4 ±1.4 nm and

Table 5. Univariate Linear Regression of Cholesterol Fractions or Extent of Lesions in Aortic Intima-Inner Media as Predictors of Arterial Influx of Cholesterol Fractions in Rabbits of the St. Thomas's Hospital Strain

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Aortic arch</th>
<th>Thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope*</td>
<td>R²</td>
<td>Slope*</td>
</tr>
<tr>
<td>LDL experiments (n=25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>104</td>
<td>0.59</td>
<td>31</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>5.6</td>
<td>0.55</td>
<td>3.0</td>
</tr>
<tr>
<td>IDL experiments (n=14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL cholesterol (mmol/l)</td>
<td>80</td>
<td>0.49</td>
<td>33</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>3.6</td>
<td>0.38</td>
<td>2.1</td>
</tr>
<tr>
<td>VLDL experiments (n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/l)</td>
<td>97</td>
<td>0.67</td>
<td>32</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>13</td>
<td>0.57</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Analyses are based on values shown in Figures 2-4.

*The units of the slopes are picomoles per square centimeter per hour per millimole per liter and picomoles per square centimeter per hour per percent lesion for lipoprotein cholesterol and lesion percentage, respectively. All 18 linear regressions were statistically significant at p<0.05. LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein.

Table 6. Multiple Linear Regression of Cholesterol Fraction Simultaneously With Percentage of Intima Bearing Lesions, as Predictors of Arterial Influx of Cholesterol Fraction in Rabbits of the St. Thomas's Hospital Strain

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Aortic arch</th>
<th>Thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope*</td>
<td>R²</td>
<td>Slope*</td>
</tr>
<tr>
<td>LDL experiments (n=25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>64</td>
<td>NS</td>
<td>64</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>2.9</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>IDL experiments (n=14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL cholesterol (mmol/l)</td>
<td>62</td>
<td>NS</td>
<td>74</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>NS</td>
<td>1.9</td>
<td>NS</td>
</tr>
<tr>
<td>VLDL experiments (n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/l)</td>
<td>38</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Intima with lesions (%)</td>
<td>NS</td>
<td>1.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Analyses are based on the values shown in Figures 2-4.

*The units of the slopes are picomoles per square centimeter per hour per millimole per liter and picomoles per square centimeter per hour per percent lesion for lipoprotein cholesterol and lesion percentage, respectively. All nine multiple linear regressions (R²) were statistically significant at p<0.025. However, six of 18 slopes did not reach independent statistical significance.

LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; NS, slope not statistically significantly different from zero (p>0.05 on one-sided t test).
The aim of the present study was to identify determinants of the rate of transfer of cholesterol from plasma LDL, IDL, and VLDL into the arterial intima. For this purpose, we employed genetically hyperlipidemic rabbits of the St. Thomas's Hospital strain. Because >95% of the apo B label remained in the LDL fraction in plasma in LDL experiments and because about 95% of the apo B label of saline extracts from intima was in the LDL fraction, influx in these experiments represents influx of LDL particles. However, when labeled IDL and VLDL were injected, about 25% and 10% of the apo B label appeared in plasma LDL and IDL during the time course of the experiments, respectively, and therefore, some of the influx in these IDL and VLDL experiments may have been due, in part, to arterial influx of IDL-derived and VLDL-derived lipoproteins. This is supported by the data in Table 4, but the quantitative contribution of IDL-derived LDL and of VLDL-derived IDL and LDL cannot be determined from these data because, on average, only 38% and 19% of the total intimal radioactivity was recovered in the apo B-containing lipoproteins isolated from the arterial samples in these validation experiments. It is likely that the remaining label is due to apo B–containing lipoproteins that are partly degraded or attached to arterial components; whether these lipoproteins entered the intima as VLDL, IDL, or LDL particles was not determined. Because influx and intimal clearance calculations were based on total arterial apo B radioactivity, the poor recovery in these validation experiments does not affect the conclusions of the present article. The results in Table 4, as well as previous results of human studies from this laboratory,

Plasma Concentration of Lipoproteins as a Determinant of Arterial Influx

The present studies suggest that arterial influx of LDL, IDL, and VLDL cholesterol in genetically hyperlipidemic rabbits is linearly and positively related to the plasma concentration of the three lipoproteins, independent of the extent of arterial lesions. However, because the square-root-transformed and the logarithmically transformed lipoprotein cholesterol influx val-

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Line plot showing comparison of intimal clearances of two lipoprotein fractions measured within the same aortic sites of rabbits 14–25; simultaneously measured intimal clearances are connected by solid lines. Intimal clearance was calculated as radioactivity in tissue (counts per minute per square centimeter), corrected for contamination, and divided by the time-averaged radioactivity concentration in plasma (counts per minute per nanoliter) and by the length of the influx period (hours).

46.8±1.2 nm for OsO4-fixed VLDL, and 56.5±2.2 nm and 55.5±2.1 nm for unfixed VLDL kept at 37°C. Because the diameters of VLDL fixed with OsO4 were considerably smaller than those of unfixed VLDL, unfixed VLDL was probably flattened during electron microscopy.23 Diameters of human LDL, IDL, and VLDL are 18–25 nm, 25–35 nm, and 30–80 nm, respectively.24

**Figure 6.** Transmission electron photomicrographs of negatively stained plasma lipoproteins from a rabbit of the St. Thomas's Hospital strain. Very low density lipoprotein (VLDL) was fixed with OsO4 before negative staining. Diameters of 100 randomly selected lipoproteins in each lipoprotein class were determined for two rabbits. LDL, low density lipoprotein; IDL, intermediate density lipoprotein.
ues were, to some extent, also associated with lipoprotein concentration in plasma and extent of aortic lesions, our data do not conclusively demonstrate a linear relation between lipoprotein concentration in plasma and lipoprotein influx into the arterial intima. Niehaus et al demonstrated a univariate, positive association between plasma LDL cholesterol and arterial influx of LDL apo B in humans, but it could not be excluded that this association reflected a simultaneous association between plasma LDL cholesterol and extent of arterial lesion. Also in support of our finding, Newman and Zilversmit demonstrated that a group of cholesterol-fed rabbits with fivefold higher plasma cholesterol levels than a group of “normocholesterolemic” rabbits had three times the arterial influx of free and esterified cholesterol compared with the normocholesterolemic rabbits; this was not a function of the extent of arterial lesion.

The linear relation between arterial influx of cholesterol in LDL, IDL, VLDL, and their product lipoproteins and plasma concentrations of LDL cholesterol in the range 0.2–6.4 mmol/l, IDL cholesterol in the range 0.1–7.0 mmol/l, and VLDL cholesterol in the range 0.7–8.5 mmol/l, respectively, suggest that nonspecific molecular sieving may be a quantitatively important mechanism for transfer of these lipoproteins into the arterial intima across endothelial cells. In keeping with this, arterial influx of LDL in normal rabbits does not depend on endothelial LDL receptors, and in cholesterol-fed rabbits, arterial influx of lipoproteins depends on lipoprotein particle size. However, from our data it cannot be ruled out that saturation kinetics for arterial influx of lipoproteins might occur at plasma concentrations of LDL, IDL, and VLDL above those examined in the present study.

Because the amount of cholesterol per VLDL-derived IDL particle is less than that per VLDL particle, arterial influx of VLDL cholesterol may have been slightly overestimated. A similar slight overestimation of arterial influx of IDL cholesterol influx, but not of LDL cholesterol influx, may have occurred. However, because it is likely that a comparable percentage of overestimation would occur in rabbits with high and low arterial influx, the relation between plasma concentration and influx, as well as the relation between lesion size and influx, would not be affected by such an overestimation.

**Extent of Arterial Lesion as a Determinant of Arterial Influx**

That the extent/severity of atherosclerosis is a determinant of influx of lipoproteins is in accordance with previous studies of cholesterol-fed rabbits, monkeys, and humans. However, the present demonstration that arterial influx of LDL, IDL, and VLDL cholesterol may be linearly dependent on the extent of arterial lesion, independent of the plasma lipoprotein concentration, has not previously been demonstrated. Schwenke and Carew have reported that in cholesterol-fed rabbits, intra-arterial concentration of LDL increases before development of fatty streak lesions, implying that the extent of arterial lesions may be determined, at least partly, by the extent of LDL influx.

**Lipoprotein Size as a Determinant of Arterial Influx**

Stender and Zilversmit demonstrated, in cholesterol-fed rabbits, an inverse relation between the particle diameters of albumin, HDL, LDL, and β-VLDL macromolecules and their arterial intimal clearance when such intimal clearances were measured within the same aortas. Thus, influx was assessed after normalization for differences in plasma concentrations of lipoproteins and for extent of arterial lesion. A similar relation between particle size and intimal clearance of HDL and LDL has been demonstrated in humans and pigs. In the previous studies cited, labeled free and esterified cholesterol was employed; our investigation, employing iodinated lipoproteins, has confirmed that such an inverse relation exists and excludes the notion that the previous demonstrations were influenced by the mathematical methods employed. The relation is true even for LDL, IDL, and VLDL, for which the differences in average diameter are only about 30%; in the earlier studies cited, the differences in diameter between the lipoprotein fractions examined were twofold to threefold. The much smaller diameter difference between the lipoproteins examined in the present study may explain the less-pronounced size dependency demonstrated in Figure 5 than that reported in the previous articles. The diameter of LDL has also been shown to be a determinant of LDL intimal clearance.

**Comparison of the Atherogenic Potential of Very Low Density Lipoprotein, Intermediate Density Lipoprotein, and Low Density Lipoprotein**

To the extent that the ability of an apo B-containing lipoprotein fraction to deliver cholesterol from plasma into the arterial intima is a measure of its atherogenic potential, our findings suggest that, at least in rabbits of the St. Thomas’s Hospital strain, IDL and VLDL may share with LDL the potential for promoting atherogenesis. This is supported by our recent article showing that IDL cholesterol and Sf 12–60 lipoprotein cholesterol were better predictors of the extent of aortic atherosclerosis in the St. Thomas’s Hospital rabbit strain than was LDL cholesterol. Although the intimal clearance of IDL and VLDL was 15–35% less than that of LDL, the cholesterol influx from the IDL and VLDL fractions may well have exceeded that from the LDL fraction in many rabbits; the cholesterol content in IDL plus VLDL was greater than that in LDL in many animals (data not shown).

In humans without major genetic forms of hyperlipidemia, there is evidence that LDL as well as IDL–8 may contribute to the atherogenic process. Furthermore, accelerated atherosclerosis in humans is a feature of 1) type III hyperlipidemia, in which Sf
12–60 lipoprotein levels (IDL plus small VLDL) are grossly elevated while LDL levels typically are low; 2) chronic renal failure and non–insulin-dependent diabetes mellitus, in each of which lipoprotein abnormalities include elevated levels of SF 12–60 lipoprotein; and 3) familial combined hyperlipidemia, in which IDL and VLDL, and/or LDL levels may be elevated.1–3

When plasma triglyceride is only moderately elevated (2–10 mmol/l) as in rabbits of the St. Thomas’s Hospital strain, in cholesterol-fed rabbits, and in the clinical disorders discussed above, particles within the VLDL–IDL classes may well be atherogenic, as such relatively small particles appear to have access to the arterial intima. In contrast, when plasma triglyceride is severely elevated, as in humans with lipoprotein lipase deficiency or apolipoprotein C-II deficiency or in alloxan-diabetic, cholesterol-fed rabbits, most lipoproteins in the VLDL fraction (d<1.006 g/ml) are large (>75 nm in diameter; SF>400 lipoprotein), and these large lipoproteins have been shown to be excluded from entering the arterial intima in diabetic, cholesterol-fed rabbits.19 Humans with lipoprotein lipase or apolipoprotein C-II deficiency, like diabetic, cholesterol-fed rabbits, appear to be relatively protected from atherosclerosis.43,44

In conclusion, our present results in genetically hyperlipidemic rabbits are compatible with a linear dependency of arterial influx of LDL-, IDL-, and VLDL-derived cholesterol on plasma concentrations of these three lipoproteins; arterial influx of lipoproteins may, therefore, involve nonspecific molecular sieving. Furthermore, to the extent that the intimal influx of apo B-containing lipoproteins is a determinant of atherogeneity, the data suggest that when plasma levels of smaller VLDL and IDL are elevated, these lipoproteins share with LDL the potential for promoting atherosclerosis.

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**KEY WORDS** • atherosclerosis • low density lipoproteins • intermediate density lipoproteins • very low density lipoproteins • familial combined hyperlipidemia • genetic hyperlipidemia • lipoprotein size • lipoprotein influx • St. Thomas's Hospital rabbit strain
Influx in vivo of low density, intermediate density, and very low density lipoproteins into aortic intimas of genetically hyperlipidemic rabbits. Roles of plasma concentrations, extent of aortic lesion, and lipoprotein particle size as determinants.

B G Nordestgaard, A Tybjaerg-Hansen and B Lewis

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