Different Efflux Pathways for High and Low Density Lipoproteins from Porcine Aortic Intima

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To study the efflux of high (HDL) and low (LDL) density lipoproteins from the arterial wall in vivo, a surgical model in pigs was used. An isolated segment of the lesion-free thoracic aorta was pulse labeled from the lumen of the artery with \(^3\)H-cholesteryl ester labeled HDL and \(^14\)C-cholesteryl ester labeled LDL. Subsequently, the labeled aortic segment was exposed to cold chase in vivo. The transfer of HDL cholesteryl ester from plasma into intima expressed as intimal clearance was three to seven times greater than that of LDL cholesteryl ester. At least 50%, but possibly as much as 95%, of the HDL cholesteryl ester that entered the arterial intima during a period of 4 hours penetrated the arterial wall beyond the internal elastic lamina. In contrast, less than 15% of the LDL cholesteryl ester that entered the arterial intima in the same period penetrated beyond the luminal layer. After 24 hours of cold chase in vivo, more than 80% of both labeled HDL esterified cholesterol and labeled LDL esterified cholesterol had disappeared from the arterial wall. Transmural profiles after 9 hours of cold chase showed that labeled HDL was present throughout the entire arterial wall, whereas labeled LDL in quantitative amounts was present only in the luminal layer. The results suggest that the most important efflux route for HDL esterified cholesterol is through the vasa vasorum and lymphatics in the outer media and adventitia, whereas LDL esterified cholesterol predominantly leaves intima via the lumen of the artery. (Arteriosclerosis 10:477–485, May/June 1990)

It is widely recognized that high plasma levels of low density lipoproteins (LDL) cause atherosclerosis in humans and that high plasma levels of high density lipoproteins (HDL) are associated with a protective effect. Both LDL and HDL particles enter the human arterial intima from plasma.1-4 The intimal clearance of HDL is larger than that of LDL.4 The cholesterol content in arterial intima without atherosclerotic lesions of 50- to 70-year-old humans is equivalent to less than 4 years of cholesterol influx.5 Thus, since the cholesterol molecule cannot be degraded by arterial tissue, the major part of cholesterol that enters arterial intima has to leave the arterial wall again.

It is unknown to what extent cholesterol leaves the arterial intima as LDL and HDL particles. It is also unknown to what extent LDL and HDL particles leave the arterial intima via the lumen of the artery or via passage through the entire arterial wall with final efflux through the vasa vasorum and lymphatics in the outer media and adventitia. These questions were addressed in the present study. We used a previously described animal model,6 where lipoprotein/arterial wall interactions were studied in normocholesterolemic pigs with nonatherosclerotic aortas.

### Methods

**Animals**

Fourteen female Danish Country Strain pigs (Ellegård Forsøgsgrise, Sarbymagle, Denmark) weighing 40 to 90 kg were used (Table 1). The experimental protocols were in accordance with Danish regulations for experiments with animals.

**Labeling of Lipoproteins**

HDL and LDL were labeled in vitro in free and esterified cholesterol with \(^14\)C-cholesterol and \(^3\)H-cholesterol. Human HDL and LDL labeled by a similar procedure have previously been shown to enter the human intima at a rate similar to in vivo labeled lipoproteins.4 The entire labeling procedure was carried out under sterile conditions. Blood (50 to 250 ml) was drawn from the pigs' internal jugular veins into one-tenth the volume of CPD-A anticoagulant (Travenol Laboratories S.A., Castlebar, Eire). The plasma and erythrocytes were stored separately at 4°C.

When both HDL and LDL were labeled (pigs 1 to 8, Table 1), 50 to 250 \(\mu\)Ci of 4-\(^14\)C-cholesterol (Amersham, Birkerod, Denmark) in 0.4 to 2.0 ml of ethanol was added to 16 to 70 ml of plasma, and 0.5 to 3.5 mCi of 1,2-(n)-\(^3\)H-cholesterol (Amersham) in 0.1 to 0.6 ml of ethanol was added to another 6 to 20 ml of plasma sample. Both preparations were incubated at 37°C for 44 to 48 hours. During this period, the enzyme lecithin-cholesterol acyltransferase incorporated \(^14\)C- and \(^3\)H-cholesterol into esterified cholesterol. To reduce the amount of labeled free cholesterol, the two preparations were subsequently each incubated for 3 to 24 hours at 37°C with an...
Table 1. Description of Experiments for Measurements of Influx into, Penetration through, and Efflux from Arterial Wall of High and Low Density Lipoproteins

| Labeled lipoproteins | Preferentially labeled with | Source | Influx period | Efflux period |
|----------------------|---------------------------|--------|--------------|--------------|
|                      | 14C-cholesterol            | 3H-cholesterol |              |              |
| HDL/LDL influx in vivo| HDL                        | LDL     | 7            |              |
| Pig 1                | Autologous                | HDL     | 5            |              |
| Pig 2                | Autologous                | LDL     |              |              |
| HDL/LDL influx/penetration/efflux | | | | |
| Pig 3                | Blood from no. 2          | HDL     | 5            |              |
| Pig 4                | From no. 1                | LDL     | 3            | 2            |
| Pig 5                | Autologous                | LDL     | 3            | 9            |
| Pig 6                | From no. 5                | HDL     | 4            | 22           |
| Pig 7                | Autologous                | LDL     | 4            | 48           |
| Pig 8                | Autologous                | HDL     |              |              |
| HDL influx/penetration/efflux | | | | |
| Pig 9                | From no. 14               | EC      | 4            |              |
| Pig 10               | Autologous                | FC      | 8            |              |
| Pig 11               | Autologous                | EC      | 4            | 72           |
| LDL influx/penetration/efflux | | | | |
| Pig 12               | Autologous                | EC      | 4            |              |
| Pig 13               | From no. 11               | EC      | 4            |              |
| Pig 14               | Autologous                | EC      | 4            | 17           |

Values are given as hours. EC=esterified cholesterol, FC=free cholesterol, HDL=high density lipoproteins, LDL=low density lipoproteins.

equal volume of stored erythrocytes. During this incubation, unlabelled free cholesterol in the erythrocytes was exchanged for labeled free cholesterol in the lipoproteins. After re-isolation from the erythrocytes, the two preparations were each adjusted with salt solutions to a final density of 1.063 g/ml and were centrifuged at 4°C at 1.58 x 10^9 g x minutes (average). The contents of the tubes were aspirated as 10 consecutive fractions, and radioactivity was determined in the fractions. LDL (d < 1.063 g/ml) were the pooled two to three upper fractions, and HDL (d > 1.063 g/ml), the pooled two to three bottom fractions without the protein pellet. LDL and HDL were dialyzed for 1 to 4 days at 4°C against repetitive changes of saline. On the day of the experiment, 3H-labeled LDL were mixed with 14C-labeled HDL and vice versa to obtain two labeled preparations for two different pigs. To each labeled preparation, autologous plasma proteins were added to ensure that the protein concentration in the labeled preparation was the same as in plasma. Plasma proteins were isolated as the density > 1.21 g/ml and were dialyzed exhaustively against saline before use. The labeled preparations were filtered through an 8 µm and a 0.22 µm filter before use.

If only HDL or LDL were labeled (pigs 9 to 11 and 12 to 14, Table 1), 3H-cholesterol or 14C-cholesterol was added to one plasma sample for incubation at 37°C as described. After exchange between lipoproteins and erythrocytes and ultracentrifugation, 19 µCi of 14C-cholesterol (or 3H-cholesterol) in 0.2 ml ethanol was added to both LDL and HDL. Labeled LDL and labeled HDL were used in two different pigs. HDL and LDL were dialyzed against saline at 4°C for 1 to 4 days. During this period, 14C-cholesterol was incorporated as free cholesterol in the lipoproteins. The addition of autologous plasma proteins and filtration of the labeled preparations were as described above.

Aliquots of preparations labeled at 37°C obtained before and after incubation in the isolated aortic segment were applied to a gel filtration column of 1% agarose (Bio-Gel A 150 m, 100 to 200 mesh; Bio-Rad Laboratories, Richmond, CA). On this column, HDL, LDL, and very low density lipoproteins (VLDL) were included, but unphysiological particles of labeled free cholesterol would elute in the void volume. However, for the four samples tested, more than 99% of the label co-eluted with the lipoproteins.

Surgical Procedure
Pre-anesthesia and anesthesia were as previously described. The surgical procedure for pulse labeling of arterial tissue in the isolated aortic segment and for the following cold chase in vivo has also been described. Briefly, through a left thoracotomy, 6 to 8 cm of the descending thoracic aorta was dissected free while intercostal arteries and other aortic branches were ligated, leaving the adventitia in situ. After an external shunt of silicone tubing was inserted cranially and caudally to the dissected aortic segment, the segment was cross-clamped at each end and thus excluded temporarily from the circulation. A small plastic tube was then inserted into the isolated aortic segment, and the segment was emptied of blood and was refilled with the above described labeled preparation (3 to 10 ml). During pulse labeling of...
arterial tissue, the pressure in the isolated aortic segment was kept at 100 cm of H₂O.

After 3 to 8 hours of pulse labeling, the isolated aortic segment was emptied and flushed with saline. The cross clamps were then released, and the normal circulation was re-established for 5 minutes. After re-application of the cross clamps, the isolated aortic segment was divided transversely and a 4 to 5 mm wide aortic ring was excised. The amount of labeled lipoproteins in this aortic ring was used to calculate the aortic intimal clearance or influx of lipoproteins, but it also represented the labeling of aorta before the in vivo cold chase period. The aorta was re-anastomosed, and the cross clamps were released to re-establish the circulation.

The animals were sacrificed by intravenous injection of 20 to 40 ml of 5% pentobarbital (DAK, Copenhagen, Denmark) at 0 to 72 hours after the operation, and the remaining part of the labeled aortic segment was excised for examination. The difference between the radioactivity in the aortic tissue removed at the operation and the radioactivity in the aortic tissue removed when the animal was terminated represents the in vivo efflux of labeled lipoproteins in the intervening period.

Immediately after excision, aortic tissue samples were rinsed thoroughly under running saline. Each aortic sample was fixed with pins on a corkboard, the area was outlined on graph paper, and the sample was divided into pieces with a luminal surface area of 0.4 to 4.9 cm². Care was taken to exclude areas that had been damaged by cross clamps, insertion of the plastic tube or re-anastomosis, and areas where intercostal arteries branched from the aorta. To ensure that the areas actually used for analysis were undamaged in terms of the transport of macromolecules, 50 to 150 ml of Evans Blue (50 μg/ml) as tracer for albumin permeability was injected intravenously into six of the pigs at 15 to 20 minutes before excision of the arterial tissue. The aortic areas inside the isolated aortic segment used for analysis were white to light blue like the rest of the arterial tree, whereas areas that had been clamped, plastic-tube inserted, or re-anastomosed were dark blue. By use of two pairs of forceps, each piece of aorta was separated into five to six consecutive layers. Each layer was weighed and stored separately at −20°C until analysis.

Microscopically, the luminal layer (layer 1) consisted of a sheet of endothelial cells directly on top of numerous elastic laminas and smooth muscle cells. The following four layers (layers 2 to 5) consisted of sheets of elastic laminas and smooth muscle cells. The adventitial layer (layer 6) had sheets of elastic laminas and smooth muscle cells in the luminal part and adventitial tissue on top of these. Vasa vasorum were observed microscopically in the four abluminal layers (layers 3 to 6).

Based on wet weights, areas, and an assumed density of 1 g/cm³ for arterial tissue, the average thicknesses of layers 1 to 6 were calculated to be 0.4, 0.3, 0.3, 0.3, 0.4, and 0.6 mm (n=14), respectively. The average protein contents of layers 1 to 6 were 0.16, 0.17, 0.15, 0.14, 0.15, and 0.10 mg/mg wet weight (n=5). The total cholesterol in layers 1 to 4 was on the average 120, 114, 86, and 111 nmol/cm² (n=9). Only 3.4% of the cholesterol in layer 1 was esterified, and for layers 2 to 4 it was only 2.5%.

**Arterial Influx of Lipoproteins In Vivo**

To compare the labeling of the arterial wall (influx of lipoproteins) in the isolated aortic segment with labeling of arterial tissue in vivo, two pigs (1 and 2, Table 1) were injected intravenously with labeled preparations containing both labeled LDL and labeled HDL. Serial blood samples were drawn over the following 5 to 7 hours until the pigs were killed, and a segment of the thoracic aorta was removed and processed as described above.

Blood containing biologically screened labeled lipoproteins was obtained from pig 2 at 10 minutes after injection of the labeled preparation. Subsequently, the blood was injected into the isolated aortic segment of pig 3.

**Analytical Procedures**

Aliquots of the labeled preparations obtained before and after incubation in the isolated aortic segments and plasma aliquots were adjusted to density 1.063 g/ml and were ultracentrifuged as described above. HDL and LDL were collected by tube slicing as the top and bottom fractions, respectively. The recoveries for ultracentrifugation were 96%±1.1% and 98%±1.0% (n=28) for ¹⁴C and ³H, respectively. To determine the cholesterol mass in VLDL (d<1.019 g/ml), other plasma aliquots were adjusted to density 1.019 g/ml and were ultracentrifuged. Extraction of lipids, separation of free and esterified cholesterol by thin-layer chromatography, and determination of radioactivity and mass of total, free, and esterified cholesterol were performed as described previously. The thin-layer chromatography recoveries of radioactivity in free and esterified cholesterol from the arterial wall were 88%±0.5% (n=298) and 92%±0.7% (n=256) for ³H and ¹⁴C, respectively. The equivalent recoveries for plasma lipoproteins were 90%±0.9% and 92%±0.9% (n=64) for ³H and ¹⁴C. Protein was determined by the method of Lowry et al. with Seronorm (Nycomed, Oslo, Norway) as a calibrator.

Total cholesterol content in plasma LDL (d<1.063 g/ml) and HDL (d>1.063 g/ml) was 1.5 and 1.2 mM (pigs 1 and 2, Table 1), with 74% and 83% of total cholesterol as esterified cholesterol in the two fractions, respectively. Of the 1.5 mM of total cholesterol in the d<1.063 g/ml fraction, only 8% was in VLDL (d<1.019 g/ml). In an additional five pigs, an average of 9% of the total cholesterol in the d<1.063 g/ml fraction was in VLDL. Therefore, in the present paper the d<1.063 g/ml fraction is designated LDL.

In the lumen of the isolated aortic segments (pigs 3 to 14, Table 1) the total cholesterol content in LDL and HDL of the installed labeled preparations were 0.6 to 4.2 mM and 0.2 to 1.5 mM, respectively. These values were similar when the labeled preparations were analyzed after the 3 to 8 hours of incubation in the isolated aortic segment.

**Calculations**

Radioactivity in an aortic tissue layer is expressed as relative tissue radioactivity, that is, the radioactivity in that layer (C_tiss, dpm/g wet weight) divided by the radioactivity...
in plasma (C_{LM}, \text{dpm/ml}). If it is assumed that the densities of arterial tissue and of plasma are both 1 g/ml, the relative tissue radioactivity is dimensionless. The depth of each aortic layer is given as a fraction of the sum of depths for the five to six consecutive layers that comprise the entire aortic wall (X/L).

The amount of labeled cholesteryl ester is named \( t_i \) (dpm x cm\(^{-2}\) x hr\(^{-1}\)) in the inner layer (layer 1) and \( t_o \) in the outer layers (layers 2 to 6). The influx of HDL and LDL esterified cholesterol from plasma into the inner layer, \( K_{pi} \) (nmol x cm\(^{-2}\) x hr\(^{-1}\)), was calculated by the “sink” method as follows. The amount of labeled esterified cholesterol in the entire arterial wall (\( t_i + t_o \)) was divided by the average cholesteryl ester specific activity in the plasma preparation in the lumen of the isolated aortic segment, \( S \) (dpm x nmol\(^{-1}\)), and by the duration of the influx period, \( T \) (hour). To take into account that \(^3\)H- and \(^14\)C-esterified cholesterol were both present in both HDL and LDL, influx of HDL and LDL cholesteryl ester was calculated with a modification of a previously published method\(^8\) with two linear equations:

\[
\frac{(t_i + t_o)}{T} = K_{p,HD} x S_{HDL} + K_{p,LDL} x S_{LDL} \quad (1, 2)
\]

written for \(^3\)H and \(^14\)C, respectively.

Intimal clearance (\( \text{nl x cm}^{-2} \text{x hr}^{-1} \)) is influx (\( \text{nmol x cm}^{-2} \text{x hr}^{-1} \)) divided by the plasma concentration (\( \text{nmol x ml}^{-1} \)) of the corresponding constituent. Equations 1 and 2 are valid if transfer of labeled cholesteryl ester out of the arterial wall (layers 1 to 6) is negligible compared with the amount of labeled cholesteryl ester that enters the wall during the influx period. In Figure 5 of the previous article,\(^6\) labeling of the arterial wall with labeled total plasma cholesteryl ester was compared for 2 and 4 hours of labeling; the “sink assumption” seemed justified since the relative tissue radioactivity of the total arterial wall after a 2-hour labeling was 51% of that after a 4-hour labeling. It is also assumed that neither hydrolysis of labeled esterified cholesterol nor esterification of labeled free cholesterol occurs in the arterial wall during labeling of the isolated aortic segment. The quantitative importance of these conversions was demonstrated in Figure 6 of the previous article\(^6\) by use of plasma labeled with \(^3\)H primarily in esterified cholesterol and with \(^14\)C primarily in free cholesterol, or vice versa. Based on the results from five animals, it was concluded that labeled free cholesterol was not esterified in the arterial wall and that labeled esterified cholesterol was not hydrolyzed to any large amount during the pulse labeling period.

An estimate of the minimal fluxes of plasma-derived HDL and LDL esterified cholesterol from the inner layer into the outer layers, \( K_{o} \) (nmol x cm\(^{-2}\) x hr\(^{-1}\)), can be obtained by a similar set of equations for \(^3\)H and \(^14\)C:

\[
\frac{t_o}{T} = K_{o,HD} x S_{HDL} + K_{o,LDL} x S_{LDL} \quad (3, 4)
\]

It is a minimal estimate since the specific activities of plasma-derived HDL and LDL cholesteryl ester in the inner layer are approximated by the specific activities in plasma, which are their maximal values. To calculate the real values of the \( K_{o}\)s, the mean specific activities of plasma-derived cholesteryl ester in the inner layer have to be substituted for \( S_{HDL} \) and \( S_{LDL} \) in equations 3 and 4.

Figure 1. The cholesteryl ester specific activities in plasma low density lipoproteins (LDL) (d<1.063 g/ml) and high density lipoproteins (HDL) (d>1.063 g/ml) from the time of intravenous injection of labeled autologous lipoproteins into a pig until the aorta was removed. • represent the fractions that originally were labeled.

These specific activities of plasma-derived cholesteryl ester in the inner layer were not determined in the present study, but they must lie between their initial value of zero and their maximal values of \( S_{HDL} \) and \( S_{LDL} \). Thus, the fluxes of HDL and LDL cholesteryl ester from the inner into the outer layers can be estimated from the following two equations for \(^3\)H and \(^14\)C, respectively:

\[
\frac{t_o}{T} = K_{o,HD} \times 0.5 \times S_{HDL} + K_{o,LDL} \times 0.5 \times S_{LDL} \quad (5, 6)
\]

Assumptions similar to those for equations 1 and 2 are also necessary for equations 3 and 4 and 5 and 6. When only HDL or LDL were labeled (pigs 9 to 11 and 12 and 13), the similar, but simpler, equations from the earlier article\(^6\) were used.

The remaining labeled cholesteryl ester (see Figure 4) was calculated as the radioactivity (dpm/cm\(^2\)) in the arterial tissue removed after cold chase divided by the radioactivity in the tissue removed before cold chase. It is assumed that radioactivity before cold chase is similar in the tissue removed and in that exposed to cold chase. This assumption has been validated previously in four pigs by using labeled whole plasma (see Figure 3 in reference 6). However, it was also validated in two pigs labeled with labeled HDL (pigs 9 and 10) and in two pigs labeled with labeled LDL (pigs 12 and 13, Table 1); the
variation of labeled lipoproteins between the aortic rings was similar to that shown in the previous article. By the simultaneous use of $^3$H- and $^{14}$C-free cholesterol and $^3$H- and $^{14}$C-esterified cholesterol in the pigs labeled only in HDL or LDL, the importance of the hydrolysis of esterified cholesterol during efflux was assessed. If, for instance, $^{14}$C was present almost exclusively in free cholesterol, any excess of $^3$H-free cholesterol as compared to $^{14}$C-free cholesterol at the end of the cold chase period can be attributed to hydrolysis of $^3$H-esterified cholesterol.

**Statistics**

The values are given as means±standard errors.

**Results**

**Labeled Lipoproteins**

In vivo (pigs 1 and 2), $^3$H-esterified cholesterol was preferentially in LDL, and $^{14}$C-esterified cholesterol was preferentially in HDL (Figure 1). A similar label distribution, or vice versa, was present when both HDL and LDL were used to pulse label the isolated aortic segment (Table 2, four left columns). Since labeled free cholesterol does not contribute significantly to labeled esterified cholesterol in the arterial wall in the present model, labeled free cholesterol was disregarded for pigs 1 to 8. When only HDL were labeled (Table 2, two middle columns) and when only LDL were labeled (Table 2, two right columns), $^3$H was present preferentially in esterified cholesterol and $^{14}$C, in free cholesterol or vice versa.

**Labeled Arterial Tissue**

When the arterial wall had been exposed for 4 hours to labeled lipoproteins from the lumen only (Figure 2, isolated aortic segment), labeled HDL esterified cholesterol was present throughout the entire arterial wall, whereas quantitative amounts of labeled LDL esterified cholesterol were present only in the most luminal layer (layer 1). In layers 2 to 5, it would have been possible to detect as little as 1% of the LDL radioactivity found in layer 1. On the other hand, when arterial tissue was labeled in vivo (Figure 2, in vivo), both labeled HDL esterified cholesterol and labeled LDL esterified cholesterol were present throughout the entire arterial wall, and for both fractions the highest concentration of radioactivity was found in the adventitial layer (layer 5). Since the arterial wall in vivo was exposed to labeled lipoproteins both from the lumen of the artery and from vasa vasorum, the difference in the labeling pattern between the in vivo situation and the isolated aortic segment probably represents contributions from vasa vasorum. Such vessels were observed microscopically in the four abluminal layers. As reported previously, labeled free cholesterol was present throughout the entire arterial wall after in vivo labeling, but only in layer 1 after labeling in the isolated aortic segment (data not shown).

In four pigs (not included in Table 1) labeled in the isolated aortic segment, labeled HDL esterified cholesterol and labeled LDL esterified cholesterol, as well as labeled free cholesterol, were present throughout the entire arterial wall, and the highest concentrations were in the most abluminal layers. The labeling pattern was similar to the pattern obtained when arterial tissue was labeled in vivo. Such labeling of arterial tissue is probably due to ligation of intercostal arteries peripheral to the origin of vasa vasorum. The four pigs with this labeling pattern were not included in the present study. Additionally, in five of the twelve pigs used for labeling of the isolated aortic segment in the present study (pigs 4, 5, 9, 11, and 12), small amounts of HDL esterified cholesterol, LDL esterified cholesterol, and free cholesterol radioactivity appeared in the most abluminal layers; this was probably also caused by insufficient ligation of vasa vasorum. Such pigs were included in the present study, but the label in layers 5 and 6 was disregarded since the topic of the present article is the fate of the lipoproteins that enter the arterial intima from the luminal side.

Based on the following observations, it was concluded that the abluminal labeling came from insufficiently ligated vasa vasorum and did not arrive from the luminal layers of the artery. First, in the pigs labeled in LDL, where labeling occurred in layers 5 and 6 (pigs 4, 5, and 12), there were no labeled LDL in layers 2 to 4, but there were large amounts in layer 1. It is difficult to envision that the label present in layers 5 and 6 should have arrived from the lumen, since it means that the lipoprotein transport in layers 2 to 4 should be much faster than in layers 1, 5,

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**Table 2. Specific Activities of Porcine Lipoprotein Preparations Used to Pulse Label Isolated Aortic Segments**

| Cholesterol | Labeled in HDL and LDL* | Labeled in HDL† | Labeled in LDL‡ |
|-------------|-------------------------|-----------------|-----------------|
|             | Before labeling | After labeling | Before labeling | After labeling | Before labeling | After labeling |
| Esterified  | LDL        | HDL        | LDL        | HDL        | LDL        | HDL        |
| $^14$C      | 915        | 83         | 902        | 53         | 44         | 271        | 1404        | 1389        |
| $^3$H       | 337        | 351214     | 457        | 34637      | 51776      | 45122      | —           | —           |
| Free        |             |             |             |             |             |             |             |             |
| $^14$C      | 10305      | 7819       | 2533       | 2607       |             |             |             |             |
| $^3$H       | 11600      | 11750      | 86444      | 90243      |             |             |             |             |

Values are dpm/nmol.

*Pigs 3 to 8. †Pigs 9 to 11. ‡Pigs 12 to 14.

HDL=high density lipoproteins, LDL=low density lipoproteins.
Intimal Clearance of High and Low Density Lipoproteins

In the isolated aortic segment, the intimal clearance of HDL cholesteryl ester was three to seven times greater than that of LDL cholesteryl ester (Figure 3). The equivalent ratios measured in vivo were two to three times greater. Due to contributions from vasa vasorum to radioactivity in layers 2 to 6 of the arterial wall in vivo as shown in Figure 2, only the radioactivity in layer 1 was used to calculate the intimal clearances in pigs 1 and 2. Similarly, only the radioactivity in layer 1 was used in the pig in which biologically screened labeled lipoproteins were injected into the isolated aortic segment (open circles); the reason for this was insufficient counts in layers 2 to 6. Thus, in these three pigs, intimal clearances probably are underestimated.

Penetration through Arterial Wall of High and Low Density Lipoprotein

Of the average 123 pmol x cm⁻² x hr⁻¹ HDL cholesteryl ester that entered layer 1 during a period of 4 hours, at least 50%, but possibly as much as 95%, penetrated the arterial wall beyond the internal elastic lamina (Table 3). In contrast, in the same period, less than 15% of the average 60 pmol x cm⁻² x hr⁻¹ of the LDL cholesteryl ester that entered the arterial intima penetrated beyond the luminal layer.

Efflux of High and Low Density Lipoproteins from Arterial Wall

After 24 hours of cold chase in vivo, more than 80% of both labeled HDL esterified cholesterol and labeled LDL esterified cholesterol had disappeared from the arterial wall (Figure 4). We have previously shown that 10% to 20% of the labeled esterified cholesterol that disappears from the arterial wall in this animal model can be
The efflux in vivo of high density lipoprotein (HDL) and low density lipoprotein (LDL) esterified cholesterol from the entire arterial wall (layers 1 to 6). The radioactivity at time 0 was the mean value of two rings of aorta for all six pigs (5 to 8, 11, and 14). The remaining labeled cholesteryl ester is shown as the means±standard errors of three to five rings of aortic tissue.

explained by hydrolysis. In the present study, in pig 11 (72-hour efflux) 23% of HDL esterified cholesterol had been hydrolyzed and in pig 14 (17-hour efflux) 12% of LDL esterified cholesterol had been hydrolyzed in the arterial wall.

After 9 hours of cold chase in vivo, labeled HDL esterified cholesterol was present throughout the entire arterial wall (Figure 5); however, labeled LDL esterified cholesterol was present in quantitative amounts only in the luminal layer (layer 1). Results similar to those in Figure 5 were also found for the five other pigs shown in Figure 4.

Discussion

Intimal Clearance of High and Low Density Lipoproteins

It has previously been demonstrated in cholesterol-fed rabbits and in humans in vivo that the intimal clearance of HDL is greater than that of LDL. Our present results show that the same is true in normocholesterolemic pigs in vivo, as well as in the isolated aortic segment. This suggests that the endothelial cell layer on aortic intima excludes LDL more effectively from entering the arterial intima than it excludes HDL, and this is in accordance with a molecular sieve-like transfer of plasma lipoproteins into the arterial wall.

Penetration through Arterial Wall of High and Low Density Lipoproteins

The present data seem to indicate that most of the HDL that enter the arterial intima go through the arterial wall, whereas only a small part of the LDL that enter the intima penetrate beyond the internal elastic lamina. This conclusion is based on two sets of observations. First, a simple two-pool model of the arterial wall, during a period of 4 hours, possibly as much as 95% of HDL esterified cholesterol but less than 15% of LDL esterified cholesterol penetrated deep into the arterial wall from the luminal layer (Table 3). Second, after a subsequent 2 to 72 hours of a cold chase period in vivo, labeled HDL esterified cholesterol was found deeper in the arterial wall, which was not the case for labeled LDL esterified cholesterol (Figure 5).

The calculations for the two-pool model (equations 5 and 6) assume that the time averages of the cholesteryl ester specific activities of the plasma-derived LDL and HDL in layer 1 during the influx period are both half of the specific activities in plasma. If LDL specific activities in layer 1 were 10% of those in plasma and HDL specific activities in layer 1 were 90% of those in plasma, 43% of the LDL that enter intima from the lumen would then have penetrated the arterial wall and 53% of the HDL would have penetrated. However, such results would not agree with the radioactivity found in the tissues after the cold chase period; more than 80% of labeled LDL had disappeared from the arterial wall (Figure 4) when still insignificant amounts of labeled LDL were found deep in the arterial wall (Figure 5, left lower column). If, on the other hand, LDL specific activities were relatively higher than HDL specific activities in layer 1 of the arterial wall, the
difference in penetration between the two particles would be even more pronounced than shown in Table 3.

In our previous article in which the use of the pig model was described and where HDL and LDL were labeled with the same isotope, we reported that 80% to 90% of the labeled plasma cholesteryl ester that had entered the arterial wall penetrated beyond the internal elastic lamina. Since the results in the previous article showed that 40% of plasma cholesterol was in HDL and because the intimal clearance of HDL is three to seven times that of LDL (Figure 3), more HDL cholesteryl ester must have entered the arterial intima than LDL cholesteryl ester. Because of that, and because the specific activity of cholesterol ester was higher in HDL than in LDL, the results of the previous paper mostly reflect the movement of HDL and therefore do not contradict the present results.

Fry and coworkers investigated the transfer of iodinated LDL and albumin from the lumen into denuded endothelialized, minipig aorta during a 24-hour incubation in vitro; this means that the transfer from the lumen directly through the internal elastic lamina was measured. They demonstrated a clearance 16 times higher for albumin than for LDL (reported as normalized uptake). This difference is in accordance with the pronounced difference between penetration of HDL and LDL through the internal elastic lamina reported in the present article, provided the penetration is dependent on particle diameter (for HDL ~10 nm, for albumin ~7 nm, and for LDL ~20 nm). Bratzler et al. found only a twofold difference in medial penetration between albumin and LDL in rabbits in vivo. However, in those two reports the arterial wall was exposed to labeled material both from the lumen of the artery and from vasa vasoorum. Radioactivity in the vasa vasoorum will diminish the estimate of a difference between LDL and albumin in medial penetration.

Based on steady-state concentration profiles of masses of LDL, HDL, and various plasma proteins across the human aortic wall, Smith and Staples suggested that the internal elastic lamina provided an almost total barrier to LDL, but not to HDL or plasma proteins. The concentration of LDL on the medial side of the internal elastic lamina was only 0.3% of its intimal concentration. The same values for HDL and albumin were 15% and 26%, respectively. Similarly, Hoff et al. were not able to detect apolipoprotein B in the media of human aortas, but found large amounts in intima.

**Table 3. Influx into and Penetration through Arterial Wall of High and Low Density Lipoprotein Esterified Cholesterol**

| Lipoproteins | Influx | Penetration |
|--------------|--------|-------------|
| HDL (n=8)†‡ | 123±51 | 59±36 (48%)§ | 117±72 (95%)§ |
| LDL (n=8)§ | 60±14 | 4±1 (7%)§ | 7±2 (12%)§ |

Values are pmol × cm⁻² × hr⁻¹ and means ± SE.

*Pigs 4 to 11. Pig 3 had insufficient counts in layers 2 to 6.

 § Pig 4 to 8 and 12 to 14. $K_n$ as a percent of $K_m$.

**Efflux of High and Low Density Lipoproteins from Arterial Wall**

The present study provides direct evidence for the disappearance of both HDL and LDL cholesteryl ester from the arterial wall (Figure 4), and only about 20% of this disappearance can be explained by hydrolysis in the arterial wall for either of the two groups of particles. Because as much as 95% of HDL esterified cholesterol may penetrate beyond the internal elastic lamina and since labeled LDL cholesteryl ester does not accumulate in the outer layers of the arterial wall, most HDL particles probably efflux from the arterial wall through the vasa vasoorum and lymphatics in the outer media and adventitia. On the contrary, because most LDL cholesteryl ester does not appear to penetrate deep into the arterial wall, most LDL particles probably leave via the lumen of the artery.

Studies by Carew et al., who used LDL labeled with 125I-tyramine-cellobiose, suggest that 70% to 75% of the LDL entering the aorta in normal rabbits leaves the arterial wall again without being degraded. This suggests an efflux of intact LDL particles from the arterial wall. In that article, it was shown that 40% of the LDL degraded by the arterial wall was degraded by endothelial cells; that is, by cells luminal to the internal elastic lamina. LDL degraded in the abluminal layers may well have entered the arterial wall through the vasa vasoorum. Compared to previous studies of arterial efflux of plasma lipoproteins in vivo, the present model has the advantages of exposing the arterial wall to labeled lipoproteins from the lumen only and of directly demonstrating the efflux of plasma lipoproteins from the arterial wall. Inherent in the advantages of the model are some disadvantages. The pulse labeling of the isolated aortic segment occurred without pulsative pressure, without oxygenated erythrocytes, and with the various intercostal arteries and vasa vasoorum ligated. However, three observations suggest that the isolated aortic segment was grossly undamaged in terms of transport of macromolecules. First, the parts of the isolated aortic segment that were used for analysis did not take up more Evans Blue dye than did the rest of the artery, suggesting a normal albumin permeability in that segment. Second, the relationship between the intimal clearances of HDL and LDL is similar whether measured in the isolated aortic segment or in vivo in pigs, in cholesterol-fed rabbits, or in humans. Third, as previously reported, the uptake of labeled free and esterified cholesterol into layer 1 of the...
arterial wall was found to be approximately the same in vivo and in the isolated aortic segment when measured in the same pig.

It seems warranted to speculate on the relevance of the present findings to atherogenesis. When plasma LDL levels are increased, it is conceivable that the flux of LDL particles into the intima is increased, and this may then lead to an increased concentration of LDL particles between endothelial cells and the internal elastic lamina and subsequently to development of atherosclerotic plaques. Increased plasma concentrations of HDL may also lead to an increased flux of HDL particles into the intima. Since the internal elastic lamina may not be as important a barrier for HDL as for LDL, the intimal concentration of LDL may not increase as much as that of LDL. Increased flux of HDL particles through the intima, however, may increase the mobilization of LDL-derived cholesterol from intima, which may explain the possible atherosclerosis-protective effect of high plasma levels of HDL.

The present data suggest that the quantitatively most important efflux route from the arterial wall for HDL is through the vasa vasorum and the lymphatics in the outer media and the adventitia, whereas LDL appear to efflux mainly via the lumen of the artery.

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