Large lipoproteins are excluded from the arterial wall in diabetic cholesterol-fed rabbits

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Abstract In diabetic hypercholesterolemic rabbits at plasma triglyceride concentrations of approximately 5000 mg/dl, 55% of plasma cholesterol (1400 mg/dl) was in lipoproteins with diameters larger than 75 nm ($S_f > 400$), 40% in smaller very low density and intermediate density lipoproteins, 4% in low density lipoproteins, and 1% in high density lipoproteins. Specific intimal clearance (nl/h · mg aortic cholesterol) of the giant $S_f > 400$ lipoproteins was about 4% of that of the low density lipoproteins. The data suggest that even very low density lipoproteins with diameters smaller than 75 nm were practically excluded from entering the arterial wall. Specific intimal clearance of low density lipoproteins in hypertriglyceridemic, diabetic cholesterol-fed rabbits was similar to that in normal cholesterol-fed rabbits, but low density lipoprotein concentrations in diabetic rabbits were low. Thus, at plasma triglyceride concentrations of approximately 5000 mg/dl, only 5% of plasma cholesterol may be readily available for infiltration of arteries. These results add further support to the hypothesis that hypertriglyceridemic, diabetic cholesterol-fed rabbits are protected against atherogenesis because the major part of plasma cholesterol is carried in large lipoproteins to which the artery is not very permeable.—**Nordestgaard, B. G., and D. B. Zilversmit.** Large lipoproteins are excluded from the arterial wall in diabetic cholesterol-fed rabbits. *J. Lipid Res.* 1988. **29:** 1491–1500.

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Diabetic rabbits that develop severe hypertriglyceridemia are protected against cholesterol-induced atherogenesis (1–4), whereas diabetic cholesterol-fed rabbits with normal plasma triglyceride develop atherosclerosis (2, 5). In severely hypertriglyceridemic diabetic cholesterol-fed rabbits, most of plasma cholesterol is carried in the large, less dense lipoproteins (3, 4, 6, 7) and less than 3% is carried in the smaller intermediate, low, and high density lipoproteins combined (6). As an explanation for the retarded development of atherosclerosis in these rabbits, it has been hypothesized that the large lipoproteins are not able to cross the arterial endothelium and consequently,

that only little cholesterol will enter into the arterial wall (7). This hypothesis is supported by the observation that the permeability coefficient or intimal clearance (nl/cm² · h) of total plasma cholesteryl ester in arteries of severely hypertriglyceridemic diabetic cholesterol-fed rabbits was reduced 50–90% compared to that in normotriglyceridemic cholesterol-fed rabbits with similar aortic cholesterol content (8). However, it is not known whether this relatively low aortic intimal clearance of cholesteryl ester is due to the largest lipoproteins being excluded from entering arterial tissue or to reduced arterial permeability to all plasma lipoproteins, i.e., that both the large $S_f > 400$ lipoproteins and the smaller very low density, intermediate density, low density, and high density lipoproteins are partly excluded from entering arteries.

To examine this question, in the present study we measured arterial uptakes of both large and smaller lipoproteins in hypertriglyceridemic diabetic cholesterol-fed rabbits and compared it to uptakes in normal cholesterol-fed rabbits.

METHODS

Animals and alloxan infusion

Two different types of influx experiments were performed. In three hypertriglyceridemic diabetic cholesterol-fed rabbits, arterial influx of lipoproteins larger or smaller than 75 nm was measured. Additionally, in

six hypertriglyceridemic diabetic and in ten nondiabetic cholesterol-fed rabbits, intimal clearance of LDL was measured. For both of these experiments, female New Zealand White rabbits (Becken Research Animal Farm, Sanborn, NY) weighing 2.6–4.4 kg were used. The rabbits were fed daily 92 g of Purina Rabbit Laboratory Chow (Ralston Purina Company, St. Louis, MO) supplemented with 0.5 g cholesterol (ICN Biochemicals, Cleveland, OH) and 7.5 g corn oil (Mazola) for 5–34 weeks prior to the influx experiments. To avoid weight loss, diabetic rabbits were fed an additional 50 g unaltered chow daily. In order to obtain hypertriglyceridemic diabetic rabbits with atherosclerotic lesions when the influx study was to be performed, four of the nine rabbits were cholesterol-fed 3–6 weeks prior to the alloxan infusion and five of the nine rabbits were injected daily subcutaneously for 3–8 weeks after alloxan infusion with 5–11 units of NPH insulin (U-100N, Lilly, Indianapolis, IN), such that plasma triglycerides were normalized.

To generate the 9 hypertriglyceridemic diabetic rabbits, 21 rabbits were infused with freshly prepared 5% alloxan monohydrate (Sigma, St. Louis, MO) in physiological saline over a period of 15 min, through a catheter inserted via the marginal ear vein. Initial doses of 150 mg/kg body weight were administered when the rabbits weighed less than 3 kg and 200 mg/kg when they weighed more. To counteract hypoglycemia, caused by insulin released from necrotic beta cells in the pancreas, the rabbits were provided with 5% sucrose instead of water for the first 24 hr. The rabbits were considered diabetic when the postabsorptive blood glucose, as determined with Dextrostix (Ames Division, Miles Laboratories, Inc., Elkhart, IN) was 250 mg/dl or more, 48 hr after alloxan infusion. Eight of the 21 rabbits did not initially develop diabetes and were reinfused with increasing doses of alloxan at weekly intervals until they became diabetic. As soon as the rabbits became diabetic, they were injected daily subcutaneously with 1–3 units of NPH insulin, which reduced the mortality within the first 3 weeks to less than 30%. Only rabbits that survived until they developed severe hypertriglyceridemia (>1000 mg/dl) and that had been cholesterol-fed long enough to develop lesions in the arteries, were used for influx experiments. Seven of the diabetic rabbits were not treated with insulin for at least the last 1–3 weeks, such that their plasma triglycerides reached higher levels prior to the influx study. One rabbit was treated daily with 3 units and one rabbit with 7 units until the experiment, but both were hypertriglyceridemic in spite of this. Unless noted otherwise, blood samples were drawn at least 15 hr after the last meal, adjusted to 0.15% Na_2EDTA and 0.4% NaN_3, and were kept on ice until plasma was separated. All experimental protocols were in accordance with University guidelines.

Validation of conditions for influx experiments

Our aim, in the present study, was to measure transfer of plasma lipoproteins into the intima of large arteries. Thus, arteries were only exposed to circulating labeled lipoproteins for 4–6 hr, such that transfer of labeled lipoproteins out of arterial intimas during the whole exposure period was small compared to arterial influx. To validate this, in two normal cholesterol-fed rabbits, arteries were exposed to circulating ^{125}I-labeled LDL for 4–6 hr and to ^{131}I-labeled LDL for approximately half the time, or vice versa.

In order to estimate the contribution of adhering plasma to radioactivity in the intima-media layer of arteries, ^{125}I-labeled LDL was injected into two hypertriglyceridemic diabetic and one nondiabetic cholesterol-fed rabbits 8 min before removal of aorta, as described below.

Aortic uptake of lipoproteins with diameters larger or smaller than 75nm

Labeling. Labeled cholesteryl ester was incorporated into lipoproteins from phosphatidylcholine–cholesteryl ester liposomes (9). This method was chosen because it is possible to label autologous lipoproteins and because label can be introduced only as cholesteryl ester without labeling of the free cholesterol fraction. [4-^{14}C]Cholesteryl oleate and [1, 2, 6, 7-[3]H(N)]cholesteryl oleate were purchased from DuPont NEN Research Products (Boston, MA). The purity was determined by thin-layer chromatography on plates precoated with silica gel (EM Science, Cherry Hill, NJ) with a hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) solvent system. Labeled cholesteryl oleate with a radiopurity less than 98% was purified by thin-layer chromatography with a hexane–diethyl ether 80:20 (v/v) solvent system and eluted from the silica gel with chloroform–methanol 9:1 (v/v).

In two screw-cap tubes, either 50 μCi [^{14}C]cholesteryl oleate or 160 μCi [^{3}H]cholesteryl oleate were mixed with 4 mg egg L-α phosphatidylcholine (Sigma) in chloroform–methanol 9:1 (v/v) and subsequently dried down in a rotary evaporator (Brinkman Instruments, Westbury, NY). After addition of 2 ml of phosphate-buffered saline (1.14% $Na_2HPO_4 \cdot 7H_2O$, 0.10% $NaH_2PO_4 \cdot H_2O$, 0.53% NaCl, pH 7.4) containing 0.01% Na_2 EDTA and 0.02% NaN_3 (PBS), the tubes were flushed with N_2, vortexed for 10 min, and sonicated for 20 min at above 51°C in a bath-type sonicator (Laboratory Supplies Company, Inc., Hickville, NY). Only suspensions that cleared were used.

Each of the two liposome preparations was mixed with 3 ml of hypertriglyceridemic diabetic plasma and subsequently incubated for 17–18 hr at 37°C. Thereafter, the two donor lipoprotein preparations were separated into lipoproteins with diameters larger and less than 75 nm ($S_f>400$ and $S_f<400$) (10) as follows. The labeled plasma preparations were each diluted to 2×4 ml in two 14×89 mm ultra-clear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA) with salt solutions to a final density of 1.063 g/ml and each was overlayered with 2 ml d = 1.041, 2 ml d = 1.019, and 4 ml d = 1.006 g/ml salt solutions. After centrifugation for $4.0 \cdot 10^6$ g-min (avg) at 21°C in a SW41 Ti rotor (Beckman Instruments, Inc.), the tubes were sliced 1.35 cm from the top (2 ml). $S_f>400$ and $S_f<400$ lipoproteins were dialyzed overnight at 4°C against two changes of 5 liters of 0.9% NaCl. The final injected donor preparations (23 ± 2 ml; n = 3) were [^{14}C]cholesteryl oleate (7 ± 1 μCi) labeled $S_f>400$ lipoproteins mixed with [^{3}H]cholesteryl oleate (38 ± 2 μCi) labeled $S_f<400$ lipoproteins; in one recipient the ^{14}C and ^{3}H were reversed.

Protocol. Immediately before intravenous injection of labeled lipoproteins into a hypertriglyceridemic diabetic cholesterol-fed rabbit, the preparation was filtered through 0.45-μm filters (Gelman Sciences, Ann Arbor, MI). After injection, blood samples were drawn at regular intervals until the rabbit was killed 4–6 hr later by intravenous injection of a 6% pentobarbital solution (50–100 mg/kg). The thorax was opened and the rabbit was perfused with 1 liter of saline introduced into the left ventricle of the heart while 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, the area was outlined on graph paper, and 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). Intima-media was stripped from the remaining media in each of these parts. From the plasma samples $S_f>400$ lipoproteins were separated from $S_f<400$ lipoproteins. Other plasma samples were adjusted to a density of 1.019 g/ml and centrifuged for at least 1.44×10^8 g-min (avg) at 10°C in a Beckman 50.3 Ti rotor. After tube slicing, the bottom fractions were readjusted to d 1.063 g/ml and recentrifuged for at least 1.44×10^8 g-min (avg) to isolate LDL and HDL as top and bottom fractions, respectively. Cholesterol from smaller VLDL + IDL was calculated as cholesterol in d<1.019 g/ml minus that in the $S_f>400$ lipoprotein fraction. Samples of aortic intima-media and plasma lipoproteins were kept at −20°C until analyzed.

Lipid analyses. Plasma triglycerides were determined by an enzymatic method (Cat. No. 701912, Boehringer Mannheim Diagnostics, Indianapolis, IN). Lipids in lipoprotein fractions were extracted into hexane from 43% ethanol in water (11). Aortic intima-media layers were minced, and the lipids were extracted during 24 hr with chloroform–methanol 2:1 (v/v) (12). Free and esterified cholesterol in the various lipid extracts were separated by thin-layer chromatography with hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) and aliquots were used for mass determination according to the method of Zak et al. (13) after saponification (14). Other aliquots were counted in toluene-based scintillant. Recoveries from thin-layer chromatography averaged 94% for lipoprotein cholesterol mass, 89% for lipoprotein radioactivities, and 97% for aortic radioactivities (bands scraped directly from thin layer chromatography plates).

Calculations. Before influx calculations, arterial radioactivities were corrected for contaminating plasma. Thus, the radioactivity in 11.0, 5.0, and 5.6 nl (see Table 1) of plasma was subtracted per cm² of arches, thoracic, and abdominal aortas, respectively.

Aortic influx of esterified cholesterol from $S_f>400$ and $S_f<400$ lipoproteins were calculated by the "sink" method, in which the amount of radioactivity in the tissue is divided by the area below the plasma specific activity versus time curve. To take into account the exchange of labeled esterified cholesterol between $S_f>400$ and $S_f<400$ lipoproteins, which occurs during the 4–6 hr influx period, a modification of a previously published method (15) with two linear equations was used:

$$t_{TC}/T = K_{EC, Sf>400} \cdot S_{EC, Sf>400} + K_{EC, Sf<400} \cdot S_{EC, Sf<400}$$

written for ^{3}H and ^{14}C, respectively;
in which:
t_{TC}, radioactivity in total aortic cholesterol [cpm/cm²];
T, influx period [hr];
K_{EC}, influx of esterified cholesterol into aorta from either $S_f>400$ or $S_f<400$ lipoproteins [nmol/cm² · hr];
S_{EC}, mean specific activity of esterified cholesterol in either $S_f>400$ or $S_f<400$ lipoproteins [cpm/nmol]. The four S_{EC}s were calculated as the time averages from such curves as those shown in Fig. 3.

Intimal clearance (nl/cm² · hr) is influx (nmol/cm² · hr) divided by the plasma concentration (nmol/nl). The intimal clearances calculated by these equations will be slightly overestimated, since labeled free cholesterol in plasma (never more than 5% of total plasma radioactivity) contributed to radioactivity in total aortic cholesterol. However, when intimal clearances were calculated with the more elaborate equations, which take radioactivity and influx rates of free cholesterol

into account (ref. 15, equations 5, 6, 7a), intimal clearances were on the average 101% of those calculated with the above equations.

Aortic uptake of low density lipoproteins

Labeling. LDL were iodinated according to the modification by Bilheimer, Eisenberg and Levy (16) of McFarlane's iodine monochloride method (17). This labeling method was chosen because iodinated apolipoprotein B in LDL is nonexchangeable. Iodine-125 and Iodine-131 were purchased from Amersham (Arlington Heights, IL).

LDL was prepared from freshly drawn rabbit blood containing the anticoagulant Na_2EDTA (2 mg/ml), the antibiotics chloramphenicol (40 µg/ml) (Sigma) and gentamycin sulfate (0.1 mg/ml) (Sigma), and the protease inhibitors ε-amino-n-caproic acid (2.6 mg/ml) (Sigma), benzamidine (10 µg/ml) (Sigma), and aprotinin (10 kallikrein units/ml) (Sigma). Sequential ultracentrifugation at 4°C was performed in solvent densities of 1.019 and 1.063 g/ml with a Beckman 60 Ti rotor for at least 2.8×10^8 g-min (avg). After ultracentrifugation, LDL was equilibrated with PBS by PD-10 gel filtration columns (Sephadex G-25M, Pharmacia, Piscataway, NJ). In aliquots (0.5–1.8 ml) containing 1–8 mg of LDL protein, pH was adjusted to 10 with glycine buffer and 1.6 to 4.0 mCi ^{125}I or 0.4 to 1.8 mCi ^{131}I was added prior to the iodine monochloride (15 nmol/mg protein), prepared as described by Goldstein, Basu, and Brown (18). Unbound iodine was removed with PD-10 columns followed by extensive dialysis at 4°C against PBS. Labeling efficiency averaged 23% and 12% for ^{125}I and ^{131}I, respectively, which converts to 1.7 and 0.9 mole of I per mole of LDL, respectively. Labeled LDL was used for influx experiments immediately after preparation, i.e., 68–92 hr after donor blood was drawn and 20–44 hr after iodination. In one animal, LDL was dialyzed for a week before the influx experiment. Injected doses were 2.7 ± 0.4 ml (n=16) with 179 ± 41 µCi ^{125}I or 44 ± 10 µCi ^{131}I.

Protocol. Immediately before intravenous injection of labeled LDL, the preparations were filtered through 0.22-µm filters (Millipore Corporation, Bedford, MA). Blood sampling, termination of the experiment, and isolation of aortic intima-media layers were performed as described above. Lipoproteins from plasma and labeled doses were isolated at 10°C by sequential ultracentrifugation at solvent densities of 1.006, 1.019, and 1.063 g/ml, each centrifuged for at least 1.44×10^8 g-min (avg.) in a Beckman 50.3 Ti rotor.

Analyses. An aliquot of the dose, plasma aliquots (200 µl), and aortic intima-media layers were counted in a Beckman Gamma 8000 counter immediately after the experiment. In order to stop enzyme reactions in these samples, 2 ml methanol was added not later than 1 hr after the samples were obtained. After counting, the same volume of chloroform was added to all samples, aortic samples were minced, and the lipids were extracted for at least 24 hr. The protein precipitate was packed by centrifugation, the solvent was removed, and the precipitate was washed twice with chloroform methanol 1:1 (v/v). To the combined washes were added half a volume of chloroform and 20% of the final volume as water. The upper phase, henceforth designated as "aqueous phase," was removed, and the lower phase ("lipid phase") was washed twice with upper phase (12). The combined aqueous phase was washed twice with chloroform. Radioactivity in the protein precipitate, the lipid phase, and the aqueous phase was determined. The distribution of label in the LDL doses in the presence of added carrier plasma between those three phases was $92 \pm 1\%$, $4 \pm 0.5\%$, and $4 \pm 0.5\%$ (n=10), respectively. The equivalent distributions for plasma samples, obtained 1 hr after injection of the dose, and aortic samples were $94 \pm 0.4\%$, $3 \pm 0.3\%$, $3 \pm 0.2\%$ (n=16) and $86 \pm 2\%$, $5 \pm 1\%$, $9 \pm 2\%$ (n=16), respectively. Aliquots of the lipid phase from aortic samples were used to determine total cholesterol mass (13) after saponification (14).

Lipoprotein fractions were dialyzed overnight at 4°C against 0.9% NaCl before counting. The distribution of label between VLDL + IDL, LDL, and HDL in the dose with added carrier plasma and in plasma obtained 1 hr and 3–4 hr after injection of the dose were $(3 \pm 1\%, 94 \pm 1\%, 3 \pm 0\%)$ (n=16), $(6 \pm 1\%, 90 \pm 1\%, 4 \pm 1\%)$ (n=16) and $(7 \pm 1\%, 88 \pm 1\%, 5 \pm 1\%)$ (n=16), respectively. In other lipoprotein fractions, lipids were extracted (11) and cholesterol was determined as described above. Aliquots of the LDL doses were electrophoresed on 1% agarose (universal electrophoresis film, Corning Medical, Palo Alto, CA); $92 \pm 1\%$ (n=16) of radioiodine comigrated with LDL. Other aliquots were delipidated (19) and apolipoproteins were separated by sodium dodecyl sulfate polyacrylamide gel (5%) electrophoresis (SDS-PAGE) (20); $89 \pm 2\%$ (n=10) of radioiodine comigrated with LDL apolipoprotein B. All the above radioiodine distribution determinations gave similar results whether ^{125}I or ^{131}I was used or whether the LDL was from a diabetic or a nondiabetic rabbit.

Calculations. Aortic intimal clearance (nl/cm² · hr) was calculated by dividing the radioactivity in the intima-media layer (cpm/cm²) by the average radioactivity in plasma during the 4–6 hr influx period (cpm/nl) and by the length of the influx period (hr).

Statistics

Values are given as means ± standard error. Linear regression analysis (ref. 21, pp. 149–193), and paired and unpaired t-tests (ref. 21, pp. 83–106) were used.

RESULTS

Plasma triglyceride and lipoprotein cholesterol

With increasing plasma triglyceride concentrations in diabetic cholesterol-fed rabbits, cholesterol content decreased in HDL, LDL, and IDL but increased in VLDL (**Fig. 1**). For HDL- and LDL-cholesterol levels, these values from an additional nine diabetic rabbits showed the same trends.
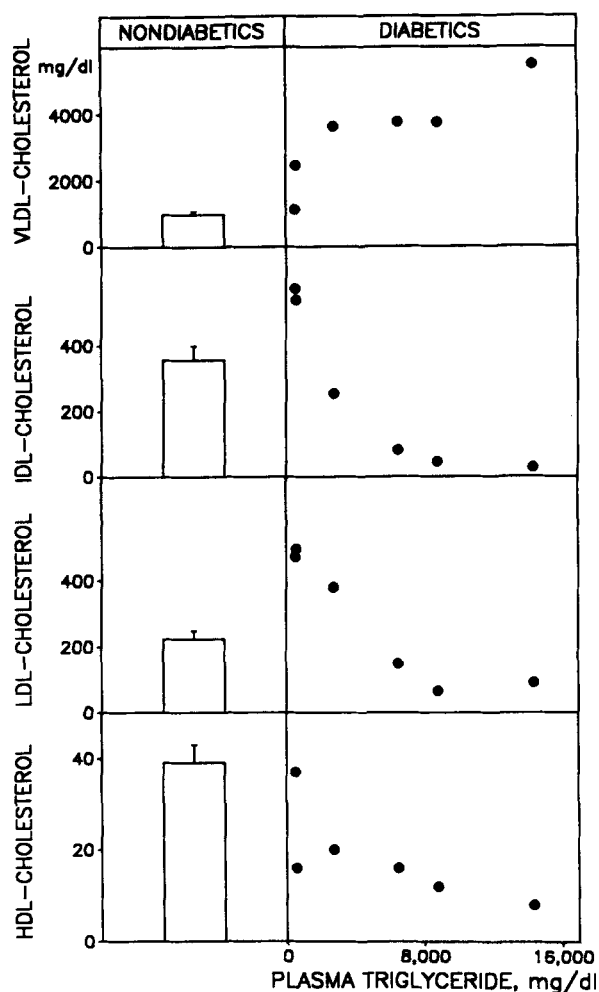

Fig. 1 Total cholesterol content in lipoprotein fractions in nondiabetic cholesterol-fed (8.3 ± 1.5 weeks) rabbits (mean ± SEM, n = 6) and in diabetic cholesterol-fed (11.3 ± 0.7 weeks) rabbits as a function of plasma triglyceride. Plasma triglyceride in nondiabetic rabbits was 94 ± 4 mg/dl.

Validation of conditions for influx experiments

In spite of up to a ten-fold difference in aortic cholesterol content, plasma contaminations expressed as nl per cm^2 of aortic surface in three rabbits were remarkably similar for the arches, thoracic aortas, and abdominal aortas (**Table 1**). In this experiment, we also measured arterial uptake of ^{131}I-labeled LDL during 4–6 hr. From 1 to 14% of the arterial radioactivity could be accounted for by plasma contamination. Corresponding corrections were made prior to influx calculations.

Accumulation of labeled LDL in intima-media of aortic arches, thoracic aortas, and abdominal aortas increased almost linearly with time for 4–6 hr (**Fig. 2**), which suggests that for these short influx periods the sink assumption is appropriate. If efflux of labeled LDL had been significant compared to influx of labeled LDL during the 4–6 hr, the six curves in Fig. 2 would have been curvilinear with a concavity toward the x-axis. Furthermore, since the two isotopes were reversed in the two rabbits, arterial uptake of ^{131}I-labeled LDL and ^{125}I-labeled LDL did not appear to differ significantly. In an additional three normal cholesterol-fed rabbits that were studied for a different purpose, isotope equivalency concerning arterial uptake was addressed directly. Two aliquots of the same LDL preparation were iodinated with ^{125}I and ^{131}I, mixed, and injected intravenously. Arterial intimal clearances of ^{131}I-labeled LDL, measured in arch, thoracic, and abdominal aortas, were 85% to 101% of those for ^{125}I-labeled LDL.

Aortic uptake of lipoproteins with diameters larger or smaller than 75 nm

An in vitro labeling technique was used to label lipoproteins with [14C]- and [3H]cholesteryl oleate, which raises the concern that some of the label might be present in a nonphysiological form, such as intact liposomes. Nonphysiological label would be taken up rapidly by the liver after intravenous injection, and the calculated volume of distribution would be much larger than plasma volume. Therefore, in two of the animals used for these influx experiments, plasma volume was determined by injecting radioactive albumin (d>1.21 g/ml), labeled biosynthetically with [3H]leucine in a normal rabbit, and the values were compared with volume of distributions for both labeled S_f>400 and labeled S_f>400 lipoproteins. Those distribution volumes for lipoproteins differed from −29% to +6% from the volumes determined by albumin. Thus, no nonphysiological label was detected. As a confirmation of findings in the earlier study (8), in the present study plasma volume was found to be expanded in hypertriglyceridemic diabetic compared to nondiabetic cho-

TABLE 1. Contamination of ^{125}I-labeled LDL (after 8 min exposure) in the intima-media layer of various aortic segments with different total cholesterol contents in cholesterol-fed rabbits

	Rabbit A	Rabbit B	Rabbit C	Mean ± SEM
Arch				
Contamination (nl/cm^2)	14.6	9.5	9.0	11.0 ± 1.8
Cholesterol (μg/cm^2)	133	54	507	
Thoracic				
Contamination (nl/cm^2)	4.5	6.5	3.9	5.0 ± 0.8
Cholesterol (μg/cm^2)	52	31	270	
Abdominal				
Contamination (nl/cm^2)	6.5	5.6	4.6	5.6 ± 0.5
Cholesterol (μg/cm^2)	55	48	370	

Rabbits A and B were hypertriglyceridemic diabetic rabbits and rabbit C was a nondiabetic rabbit. Arch: heart to first intercostal arteries. Thoracic aorta: to the diaphragm. Abdominal aorta: to the bifurcation. Contamination was calculated as radioactivity in tissue (cpm/cm^2) divided by radioactivity concentration in plasma (cpm/nl).

lesterol-fed rabbits; 51 ± 3 (n = 9) and 36 ± 2 (n = 10) ml/kg body weight, respectively (P<0.001). However, expanded plasma volume does not affect measurements of intimal clearance.

Although [^{3}H]- and [^{14}C]cholesteryl esters 10 min after injection of the dose were present almost exclusively in either the S_f<400 or S_f>400 lipoproteins, exchange of labeled esterified cholesterol occurred during the 5-hr influx period, such that the specific activities of cholesteryl ester in the two complementary lipoprotein fractions approached each other (**Fig. 3**). Since in each experiment the time-averaged specific activities for both [^{3}H]- and [^{14}C]cholesteryl ester were different for S_f>400 and S_f<400 lipoproteins, arterial influx and intimal clearance of cholesteryl ester from the two lipoprotein fractions could be calculated.

Aortic intimal clearance of cholesteryl ester from lipoproteins with diameters larger than 75 nm (S_f>400) was small (**Table 2**), and in each of three different tissues in three different hypertriglyceridemic diabetic cholesterol-fed rabbits, it was less than the intimal clearance of cholesteryl ester from lipoproteins with diameters less than 75 nm (S_f<400).

Aortic uptake of low density lipoproteins

After intravenous injection of iodine-labeled LDL, radioactivity in total plasma decreased at a similar rate in diabetic and nondiabetic cholesterol-fed rabbits, and after 4–6 hr 78 ± 3% (n = 6) and 80 ± 2% (n = 10) of the initial radioactivity remained in plasma in the two groups of rabbits, respectively. When arterial intimal clearances of LDL were calculated based on protein precipitable radioactivity, the values were 92% ± 1% (n = 35) of the values calculated based on total radioactivity in plasma and intima-media. The reported values are based on total radioactivity.

Specific intimal clearance

Aortic intimal clearances are "normalized" influx values, in which differences in plasma concentration of the various fractions are taken into account. Thus, clearances are directly comparable between different lipoprotein fractions within the same aortic tissue sample, such as those in Table 2. However, lipoprotein clearances between aortic tissues from different animals are not always comparable, since the magnitude depends not only on the lipoproteins but also on the extent of atherosclerotic lesions (22–26). Only when the aortic tissues have similar cholesterol content is it reasonable to compare lipoprotein clearances among different animals. Aortic cholesterol varied greatly among the different rabbits in the present study.

Intimal clearances of LDL in normal cholesterol-fed rabbits are directly proportional to aortic cholesterol content of aortic arches and abdominal aortas (**Fig. 4**). Although the correlation for the thoracic aorta was not very strong and only of borderline significance (P<0.1), values from an additional six rabbits studied for a different purpose, when combined with these data, increased the correlation coefficient to 0.69 and the significance level to P<0.01. Because of this relationship, we define a new term, specific intimal clearance (nl/hr • mg cholesterol), as the intimal clearance (nl/cm^2 • hr) divided by the cholesterol content in the intima-media (mg/cm^2). Arterial uptake of lipoproteins expressed in this manner is thus normalized for differences in lipoprotein concentration of both plasma and aortic cholesterol content, which makes possible comparisons among different rabbits. Similar calculations have been used previously (22,27).

All intimal clearances of lipoproteins in the present report have been recalculated as specific intimal clear-
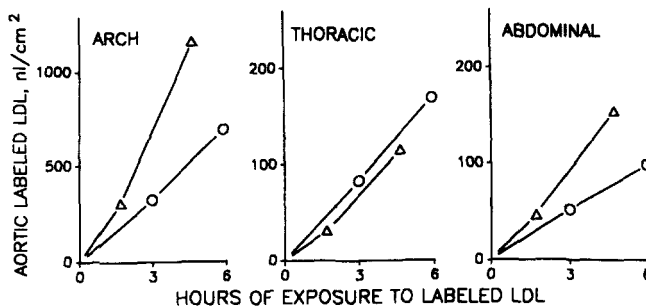

Fig. 2. Accumulation of labeled LDL (nl/cm^2) in aortic intima-media layers of normal cholesterol-fed rabbits; calculated as radioactivity in tissue (cpm/cm^2) divided by average radioactivity in plasma (cpm/nl). In one animal (△), ^{125}I-labeled LDL was injected at zero time whereas 3 hr later the same LDL labeled with ^{131}I was injected. In the other rabbit (○), the isotopes were reversed. The y-axis scale for aortic arches is five times that for the thoracic and abdominal aortas.
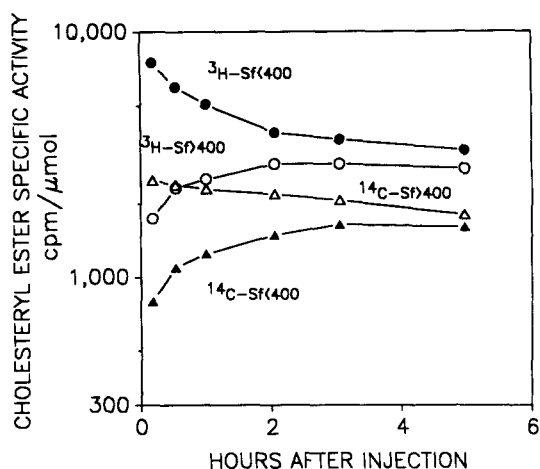
Fig. 3. Specific activities of esterified cholesterol in $S_f>400$ lipoproteins and in the combined fraction of smaller VLDL, IDL, LDL, and HDL ($S_f<400$ lipoproteins) from the time of injection of the dose until the aorta was removed. The dose was $S_f<400$ lipoproteins labeled with [^{3}H]cholesteryl ester mixed with $S_f>400$ lipoproteins labeled with [^{14}C]cholesteryl ester. The time-average of the four specific activity curves was used for influx calculations.

VLDL, IDL, LDL, and HDL (lipoproteins with diameters <75 nm). It is noteworthy that similar conclusions can be drawn whether specific intimal clearances were measured in tissues with high aortic cholesterol contents (arches) or in those with low cholesterol contents (thoracic and abdominal aortas). Specific intimal clearance of iodinated LDL has been measured in an additional four hypertriglyceridemic diabetic cholesterol-fed and six normal cholesterol-fed rabbits, studied for a different purpose. When these results are combined with those of Fig. 5, the standard errors are, on the average, reduced by 30% in diabetic rabbits and 14% in nondiabetic rabbits. Furthermore, the mean values for the two groups approach each other.

ances and shown in **Fig. 5.** Specific intimal clearance of lipoproteins with diameters larger than 75 nm was on the average 4% of that LDL. Specific intimal clearance of LDL was not significantly different between hypertriglyceridemic diabetic and nondiabetic rabbits, and was 4- to 11-fold that of the combined smaller

DISCUSSION

The aim in the present study was to investigate in greater detail the mechanism whereby severely hypertriglyceridemic diabetic cholesterol-fed rabbits are protected against atherosclerosis. Our results demonstrate that the largest lipoproteins (diameter >75 nm) in these rabbits are practically excluded from entering the arterial wall and that the much smaller LDL are taken up at a "normal" rate. Thus, the arterial wall in the hypertriglyceridemic diabetic cholesterol-fed rabbits does not appear to have an altered perme-

TABLE 2. Lipoprotein lipids in plasma and transfer of lipoprotein cholesteryl ester into aortas of hypertriglyceridemic diabetic cholesterol-fed rabbits

Rabbit No.	Plasma					Tissue	Aortic Intima-Media Layer		
	Triglyceride	Total Cholesterol					Total Cholesterol	Intimal Clearance of Cholesteryl Ester	
		$S_f>400$	Smaller VLDL + IDL	LDL	HDL			$S_f>400$	Smaller VLDL + IDL + LDL + HDL[a]
	mg/dl	*mg/dl* (esterified cholesterol as a percent of total cholesterol)					$\mu g/cm^2$	$nl/cm^2 \cdot h$	
1[b]	3470	1588 (83%)	1371 (77%)	121 (68%)	22 (62%)	Arch	2408	11.6	70.2
						Thor	1194	2.0	27.0
						Abd	618	2.4	16.0
2[c]	3190	448 (79%)	795 (71%)	132 (61%)	15 (65%)	Arch	1583	22.4	50.6
						Thor	207	2.2	7.7
						Abd	166	3.9	5.1
3[b]	8760	2111 (78%)	846 (70%)	48 (62%)	15 (45%)	Arch	1684	0.3	49.2
						Thor	277	0.0	8.9
						Abd	418	0.6	15.1
Mean	5140	1382	1004	100	17				

Intimal clearance is calculated by dividing the influx ($nmol/cm^2 \cdot hr$) by the plasma concentration ($nmol/nl$) of the corresponding constituent.
[a] $S_f < 400$ lipoproteins.
[b] The injected dose was autologous lipoproteins labeled with [^{14}C] cholesteryl ester in $S_f > 400$ lipoproteins and with [^{3}H] cholesteryl ester in $S_f < 400$ lipoproteins.
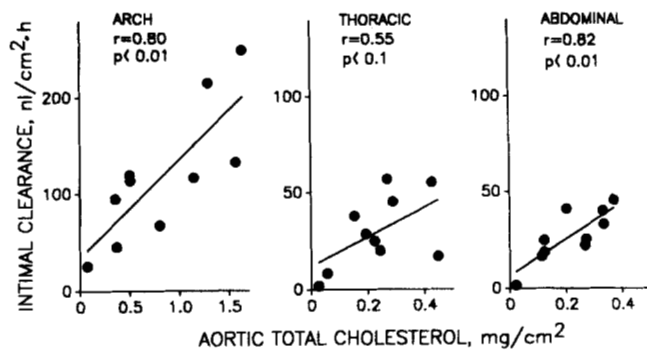[c] The injected dose was lipoproteins from rabbit no.1 with the isotopes reversed.

Fig. 4. Correlation between total cholesterol content in intima-media and intimal clearances of LDL in various aortic segments of normal cholesterol-fed rabbits. The correlation coefficients (*r*) and the significance levels (P<) for the correlations are shown for each tissue. None of the three intercepts on the y-axis was significantly different from zero. Note that the scales for aortic arch values are different from those for thoracic and abdominal aortas.

ability to lipoproteins, per se, as compared to the arterial wall in normal cholesterol-fed rabbits. Specific intimal clearance of VLDL with diameters less than 75 nm was not measured directly, but in a combined fraction together with IDL, LDL, and HDL. If HDL and LDL cholesteryl ester is assumed to be cleared by arteries at the rate measured directly for iodinated LDL, specific intimal clearance of smaller VLDL + IDL in hypertriglyceridemic diabetic cholesterol-fed rabbits can be estimated to be 6% of that of LDL.

Whether it is the large size of the $S_f > 400$ lipoproteins and possible smaller VLDL or some other property of the lipoproteins, e.g., altered apoprotein composition (4), that is the responsible factor causing exclusion from the arterial wall cannot be stated with certainty. In favor of the size being responsible is the observation that albumin, HDL, LDL, and VLDL in normal cholesterol-fed rabbits show decreasing transendothelial transport with increasing diameter of the particle in capillaries (28) and aorta (29). This suggests that transport across the arterial intimal layer is by a mechanism of nonspecific molecular sieving, dependent on the relative size of the macromolecular particles to the "pore" sizes of the blood/artery interface.

In order to compare our results with previous ones (8), the values in each set of experiments have been recalculated such that direct comparison is possible. In the earlier study (8), in vivo labeled lipoproteins were used to measure aortic intimal clearance of cholesteryl ester from all plasma lipoproteins combined into the intima-media of aortic arches in eight hypertriglyceridemic, diabetic cholesterol-fed rabbits. Aortic cholesterol content ranged from 60 to 1400 μg/cm², and the influx period was 4–6 hr. The recalculated specific intimal clearance (radioactivity equivalent of plasma contamination was subtracted) is 24 ± 10

nl/hr • mg cholesterol. Our present results were recalculated as an average specific intimal clearance for all plasma lipoproteins combined, and the values are 17 $\pm$ 5, 13 $\pm$ 3, and 18 $\pm$ 5 nl/hr • mg cholesterol for arch, thoracic, and abdominal aortas, respectively. Thus, aortic specific intimal clearance measured with in vivo labeled lipoproteins in diabetic, male Danish Country strain rabbits seem to agree with those measured in diabetic, female New Zealand White rabbits with liposome-labeled lipoproteins.

It seems of interest to compare the specific intimal clearances in the present study with those that can be calculated from Table 3 in the report by Stender and Zilversmit (29). In that study, intimal clearance of in vivo labeled lipoproteins into the intima-media of arch and thoracic aorta was measured in normal cholesterol-fed rabbits. The range of aortic cholesterol con-
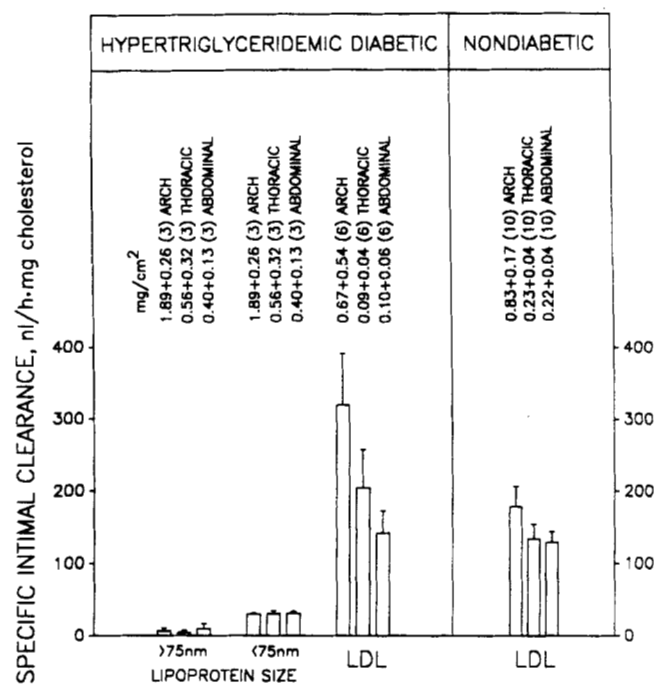

Fig. 5. Specific intimal clearances of various lipoprotein fractions by intima-medias of various aortic segments of hypertriglyceridemic diabetic and nondiabetic cholesterol-fed rabbits. Diabetic lipoproteins were injected into diabetic rabbits and nondiabetic lipoproteins into nondiabetic rabbits. Values for lipoproteins larger or smaller than 75 nm in diameter are calculated from Table 2. The results are expressed as means ± standard error. The arterial cholesterol contents (mg/cm²) are shown in the figure and in parentheses is shown the number of rabbits. Plasma triglyceride averaged 5800 mg/dl (range 1,000–16,000) in diabetic rabbits and 115 mg/dl (range 25–450) in nondiabetic rabbits. With paired *t*-tests, the differences in specific intimal clearance of lipoproteins larger or smaller than 75 nm were significant at P<0.02 for aortic arches and thoracic aortas and nonsignificant for abdominal aortas. With unpaired *t*-tests, specific intimal clearance of LDL was not different between hypertriglyceridemic diabetic and nondiabetic cholesterol-fed rabbits.

tent (70–3900 $\mu g/cm^2$) and the duration of the influx period (4–6 hr) were the same as in the present study. The recalculated specific intimal clearance of LDL protein is 194 ± 44 and of cholesteryl ester from LDL and HDL combined 235 ± 32 nl/hr • mg cholesterol. These values are in close agreement with our present findings for specific intimal clearance of iodinated LDL. Furthermore, recalculated specific intimal clearances of VLDL + IDL in the earlier study are 167 ± 22 and 115 ± 19 nl/hr • mg cholesterol for protein and cholesteryl ester label, respectively. This is less than the specific intimal clearance of LDL but much larger than that of both S_f>400 lipoproteins and S_f<400 VLDL in hypertriglyceridemic, diabetic cholesterol-fed rabbits.

It is known that the cholesterol content of the most dense lipoprotein fractions, i.e., IDL, LDL, and HDL, is reduced in hypertriglyceridemic, diabetic cholesterol-fed rabbits compared to that in normotriglyceridemic cholesterol-fed rabbits (3,4,6,7). The present demonstration of an inverse relationship between the magnitude of plasma triglyceride and HDL-, LDL-, and IDL-cholesterol in diabetic rabbits has not been reported previously. Since in the hypertriglyceridemic diabetic rabbits, only HDL- and LDL-cholesterol (possibly also IDL-cholesterol) appear to be readily available for uptake into the arterial wall, increasing plasma triglyceride appears to be associated with a decreasing fraction of plasma cholesterol being readily available for infiltration of arteries. Furthermore, since cholesterol influx into the arterial wall probably is necessary for development of atherosclerosis, this fits well with the previously reported inverse relationship between plasma triglyceride (serum neutral fat) and development of atherosclerosis in diabetic rabbits (2).

Our present results add further support to the hypothesis that hypertriglyceridemic, diabetic cholesterol-fed rabbits are protected against atherogenesis because the major part of plasma cholesterol is carried in lipoproteins too large to pass through the arterial endothelium (7). That the few VLDL + IDL particles that do enter the artery, are only relatively slowly taken up by macrophages (4) may further explain the reduced atherogenesis in these rabbits. This animal model appears to demonstrate that when cholesterol influx into arteries is drastically reduced, in spite of high plasma cholesterol levels, atherogenesis is prevented.

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