Effects of stigmastanyl-phosphocholine (Ro 16-6532) and lovastatin on lipid and lipoprotein levels and lipoprotein metabolism in the hamster on different diets[1]

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Abstract Previous studies from our laboratory have shown that oral administration of stigmastanyl-phosphocholine (Ro 16-6532) reduces plasma cholesterol levels in experimental animals on diets free of added cholesterol. In the present study, effects of Ro 16-6532 and lovastatin on lipoprotein levels and metabolism were investigated in male golden Syrian hamsters. In hamsters fed a standard diet, Ro 16-6532 (1 mmol/kg/day) lowered cholesterol in all lipoprotein fractions, as well as apoB-100 and apoA-I. In contrast, lovastatin (25 μmol/kg/day) lowered high density lipoprotein (HDL)-cholesterol but had no effect on low density lipoprotein (LDL)-cholesterol or on apoB-100 or apoA-I while triglycerides and very low density lipoprotein (VLDL)-cholesterol increased. In hamsters fed a coconut fat-supplemented diet, Ro 16-6532 reduced all lipoproteins, with a stronger effect on VLDL- and LDL- than on HDL-cholesterol. Also apoB-100 was reduced. Lovastatin (50 μmol/kg/day) reduced LDL-cholesterol, HDL-cholesterol, and apoA-I while triglycerides and VLDL-cholesterol increased. The drop in LDL-cholesterol seen with both drugs in hamsters fed the diet supplemented with coconut fat occurred without any effect on the plasma removal rate of homologous LDL, or on the content of hepatic LDL-receptors. In contrast, the first phase of removal of homologous radioiodinated VLDL from plasma was markedly increased by both compounds, paralleled with an increased uptake of label in the liver and a decreased appearance of labeled apoB-100 in the LDL-fraction. Furthermore, retinyl ester-labeled chylomicrons were also cleared more rapidly in hamsters treated with Ro 16-6532. Hepatic uptake of label from VLDL and chylomicrons was strongly decreased by pre-injection of lactoferrin. In addition, Ro 16-6532 slightly decreased the secretion rate of VLDL in hamsters fed the coconut fat-supplemented diet. Taken together, these results indicate that the reduction of LDL-cholesterol after treatment with Ro 16-6532 and lovastatin observed in the hamster is mainly due to decreased conversion of VLDL into LDL, consequent to an increased hepatic removal of VLDL remnants. Ro 16-6532 also increased the liver uptake of chylomicron remnants. The hepatic uptake system implicated in this remnant removal can be completely blocked by lactoferrin. The nature of this uptake system is still unknown.–**Himber, J., B. Missano, M. Rudling, U. Hennes, and H. J. Kempen.** Effects of stigmastanyl-phosphocholine (Ro 16-6532) and lovastatin on lipid and lipoprotein levels and lipoprotein metabolism in the hamster on different diets. *J. Lipid Res.* 1995. **36:** 1567–1585.

Supplementary key words hepatic LDL receptor · hepatic lipoprotein uptake · VLDL · LDL · HDL · apoB-100 · apoA-I · plasma lipoprotein turnover · VLDL secretion

The origin of this work was the search for improved inhibitors of cholesterol absorption, as potential new drugs for lowering plasma and LDL cholesterol levels. Plant sterols such as β-sitosterol are known to inhibit cholesterol absorption and to reduce plasma cholesterol levels in humans (1). β-Stigmastanol has been shown to inhibit cholesterol absorption more efficiently than β-sitosterol (2) and to lower serum cholesterol in hypercholesterolemic patients at relatively low doses without being absorbed (3).

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HMG, 3-hydroxy-3-methylglutaryl; apo, apolipoprotein; FCR, fractional catabolic rate; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Me-LDL, methylated LDL.

[1]Parts of these studies were presented at the XI International Drugs Affecting Lipid Metabolism meeting (8), and at the 8th (7), 9th (9), 10th (80) International Atherosclerosis Society meetings.

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In an attempt to increase the micellar solubility of this plant sterol, we synthesized stigmastanyl phosphocholine (Ro 16-6532), a sterol-phospholipid hybrid in which the β-stigmastanol hydroxyl group is esterified with phosphocholine (4). Ro 16-6532 has several biophysical properties in common with phosphatidylcholine: it forms mixed micelles with bile salts and, under certain conditions, also multilamellar liposomes similar to those of phosphatidylcholine (5, 6). As expected, oral administration of Ro 16-6532 strongly decreases intestinal cholesterol absorption in several animal models (7, 8). Strikingly, it is able to reduce plasma cholesterol in squirrel monkeys and in Watanabe heritable hyperlipidemic × Burgundy hybrid rabbits (7), as well as in hamsters (9) fed regular chow or a diet enriched in saturated fats but without added cholesterol. In contrast, other inhibitors of cholesterol absorption did not lower plasma cholesterol in hamsters fed such diets (8) or in humans (10), suggesting that Ro 16-6532 may lower plasma cholesterol by another mechanism than by decreasing cholesterol absorption.

To better understand how Ro 16-6532 reduces plasma cholesterol, the effects of oral administration of Ro 16-6532 on lipoprotein plasma levels and metabolism were evaluated in hamsters fed either a normal chow diet or the chow diet supplemented with coconut fat without or with 0.12% added cholesterol. For comparison, we studied the effects of lovastatin in hamsters fed these same diets.

It was found that Ro 16-6532 and lovastatin lower LDL-cholesterol mainly by increasing the uptake of the VLDL remnants by the liver (shunt pathway), leading to decreased conversion of VLDL into LDL, whereas clearance of LDL from plasma and the amount of LDL-receptors in liver membranes were not affected.

MATERIALS AND METHODS

Chemicals

PAGE reagents were from Bio-Rad. Molecular standards for gel calibration, 17α-ethinyl estradiol, and DL-dithiothreitol were from Sigma. Albumin bovine- fraction V, Triton WR-1339, and lactoferrin were purchased from Serva. Intralipid® 10% was from Kabi-Vitrum (Stockholm, Sweden). Octyl-β-D-glucoside and Triton X-100 were obtained from Boehringer Mannheim. $Na^{125}I$ was from Medipro AG (Teufen, Switzerland) and [^{3}H]vitamin A was from Amersham. Lovastatin was purchased as 20-mg tablets from a retail pharmacy and the pills were ground and mixed with the hamster chow. All other chemicals were of analytical grade. Ro 16-6532 was a gift from Dr. J-M. Cassal, Hoffmann-La Roche AG, Basel, Switzerland.

Animals, diets

Male golden Syrian hamsters (FUME SPF) from BRL (Füllinsdorf, Switzerland), weighing 120–140 g were housed individually in Makrolon® cages on wood-chip bedding and soft paper as nesting material with alternating periods of light (6:00–18:00) and darkness (18:00–6:00). Animals had free access to standard rodent chow (Kliba no. 343 in pellet form, Kaiseraugst, Switzerland) for at least 1 week. Special batches of this rodent chow, having either the standard composition (containing 4 wt% fat) or a composition (containing about 16 wt% fat) in which 18 wt% of the total diet was coconut kernel instead of cereals, were obtained in powdered form (also from Kliba, Kaiseraugst, Switzerland). The macro-nutrient compositions of the standard or coconut-supplemented diets are given in **Table 1.**

Before starting drug administration, animals were placed in individual cages, and acclimated to being served 10 g of the selected diet per day for a period of 7 days. For this purpose, the powdered diets were thoroughly mixed with an equal amount (by weight) of water to give a paste. When the diet should contain added cholesterol, the required amount of crystalline cholesterol was brought into suspension in water with a Potter homogenizer and this suspension was mixed into the paste. Twenty grams of this paste was served each day at 7 AM in a cup placed on the bottom of the cage. At the end of this pretreatment period, the animals consistently ate more than 90% of their daily serving. This pretreatment period was sufficient to reach a new steady state of the plasma lipids when the diet was shifted from normal chow to fat-supplemented chow (see Fig. 4). Animals were then assigned to treatment groups, such that the groups had approximately identical average body weights. For drug administration, the hamsters were maintained on the same diet as in the pretreatment period but now Ro 16-6532 or lovastatin was mixed into the slurry, such that the daily doses were 1 mmol/kg per day for Ro 16-6532 and between 25 and 190 μmol/kg

TABLE 1. Composition of hamsters' diets

Ingredients	Standard Chow	Coconut-Supplemented Chow[a]
	g/100 g anhydrous mix	
Protein	19.2	19.0
Fibre	4.8	5.6
Fat	4.1	16.4
Carbohydrate	54.3	42.6
Dietary energy (MJ/kg)	12.3	14.7
Metabolic energy in fat (%)	12	40

Both diets contain 0.006 wt% cholesterol. If indicated, some experiments were also performed with a coconut fat supplemented diet containing 0.12 wt% cholesterol (see Results).

[a]Consists of dried coconut kernel (18 wt%) instead of cereals.

per day of lovastatin. Drug administration was maintained for 14 days. Food consumption and body weight were monitored daily throughout the treatment period, and were not significantly affected by the drugs in the doses selected. However, there was a trend for less food intake with the highest dose of lovastatin. At the last day of the pretreatment period and at the 14th day of drug treatment, blood samples (200 µl) were taken via the jugular vein while the animals were under light halothane anesthesia, for determination of the plasma lipids and lipoprotein levels. Blood sampling was done between 7:30 and 8:30 AM.

Male Wistar rats (Albino-SPF also from BRL, Füllinsdorf, 100–230 g) were injected subcutaneously for 5 days with 17α-ethinyl estradiol (5 mg/kg) dissolved in propylene glycol, or with propylene glycol only, for LDL-turnover studies and for quantitation of LDL-receptors in liver membranes, as described under Results.

Isolation of lipoproteins, labeling of chylomicrons

Lipoproteins were isolated from EDTA plasma obtained from hamsters that had been fed standard rodent pellets. VLDL was obtained by centrifugation of 1 ml plasma at its own density in each of 10 polycarbonate tubes in a TL-100 Beckmann ultracentrifuge at 440,000 *g* for 30 min at 10°C in a TLA-100.2 rotor. The top layer was harvested by tube slicing (CentriTube Slicer™, Beckmann) and the pooled VLDL was washed once by reflotation of 500 µl VLDL in 500 µl 0.15 M NaCl/0.27 mM EDTA, pH 7.4, in each centrifugation tube for 90 min.

Hamster LDL was isolated as previously described (11) by a 30 min single-spin density-gradient ultracentrifugation at 440,000 *g*, 10°C, as follows. In each of 10 thick-walled polycarbonate tubes fitting in the TLA-100.2 rotor, 570 µl plasma was brought to a density of 1.21 g/ml with solid KBr and overlayered with 550 µl 0.15 M NaCl/0.27 mM EDTA, pH 7.4. In order to visualize the lipoprotein fractions, 10 µl of 0.1% Sudan Black dissolved in ethylene glycol was added to the plasma sample of one of the tubes. Lipoproteins were separated by ultracentrifugation in a TLA-100.2 rotor and LDL was sliced out of the non-prestained tubes at the height where the LDL band occurred in the tube with the Sudan-stained sample. The pooled LDL bands were then brought to a density of 1.12 g/ml with solid KBr, and 500 µl LDL was floated through a 500-µl layer of 0.15 M NaCl/0.27 mM EDTA, pH 7.4, by centrifugation for 90 min in the TLA-100.2 rotor. The top layer containing the purified LDL was free of albumin contamination as judged by SDS-PAGE (**Fig. 1**).

Methylated LDL (Me-LDL) was prepared by treatment of the LDL fraction with formaldehyde plus sodium borohydride as described (12). Rabbit β-migrating VLDL was isolated by ultracentrifugation of serum obtained from rabbits that had been on a cholesterol-enriched diet (1% cholesterol) for at least 3 weeks (13).

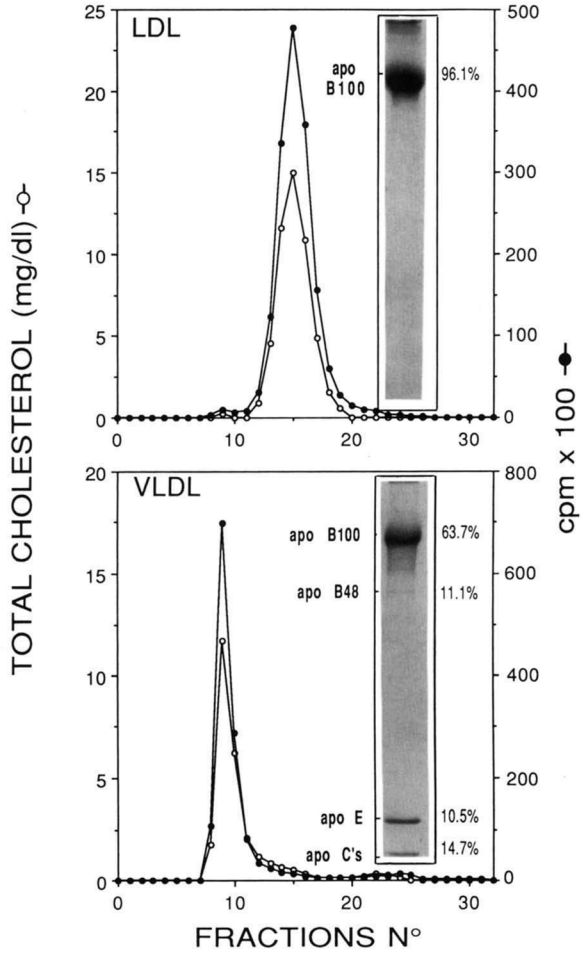

Fig. 1. Typical elution profile for the LDL fraction (upper panel) and the VLDL fraction (lower panel) isolated by discontinuous density gradient ultracentrifugation and flotation. An aliquot of 20 µl of the lipoprotein fraction (LDL or VLDL) containing a trace of ^{125}I-labeled lipoprotein from the same preparation as a radiomarker was applied to a Superose 6 FPLC column. The cholesterol concentration and the ^{125}I-radioactivity were measured in each 50-µl elution fraction. The same LDL and VLDL fractions were subjected to 3–15% gradient SDS-PAGE and exposed for autoradiography (inserts). Values in percent represent the distribution of incorporated radioactivity among the apolipoproteins of the LDL and VLDL fractions.

Hamster chylomicrons were labeled in vivo with [^{3}H]retinol and were collected from the thoracic duct (14). Briefly, 250 µCi of solvent-free [^{3}H]retinol was dissolved in 2 ml of a 10% lipid emulsion (Intralipid®) and was given by gastric intubation to hamsters 1 h before they were anesthetized with pentobarbital. After anesthesia was established the thoracic duct was cannulated. Lymph was collected for 3 h (about 600 µl) on ice in 1.5-ml Eppendorf tubes containing 0.2% EDTA. The pooled lymph from three animals was floated by ul-

tracentrifugation (at 440,000 *g* for 30 min, 10°C, Beckmann TLA-100.2 rotor). Chylomicrons were isolated by tube-slicing and 500 µl chylomicrons per tube was washed by overlayering 500 µl of 0.15 M NaCl/0.27 mM EDTA, pH 7.4, followed by centrifugation for 2 h. More than 90% of the [^{3}H]retinol was esterified and recovered in the chylomicron fraction.

^{125}I-labeling of lipoproteins

VLDL, β-VLDL, and LDL were radioiodinated using the iodine monochloride method of McFarlane (15) as modified by Bilheimer, Eisenberg, and Levy (16). Free ^{125}I was removed by Sephadex G-25 gel filtration (prepacked columns PD-10, Pharmacia) and the labeled lipoproteins were extensively dialyzed against 0.15 M NaCl/0.27 mM EDTA, pH 7.4, at 4°C with repeated changes of the buffer.

The ^{125}I-labeled LDL or ^{125}I-labeled Me-LDL preparations were used directly for the turnover studies (after dilution in 0.15 M NaCl to the desired protein concentration), as 96% of the protein-radioactivity is associated with apoB-100 (Fig. 1). The ^{125}I-labeled VLDL fraction was incubated for 60 min at 37°C in VLDL-free hamster plasma and re-isolated by tube slicing after flotation for 120 min at 10°C in a TLA-100.2 rotor (440,000 *g*). By this method an exchange occurs between cold and labeled apoE and apoCs, leading to a relative increase in radioactivity of apoB-100. In a representative VLDL preparation obtained in this manner, 63.7% of the radioactivity was associated with apoB-100, 11.1% with apoB-48, 10.5% with apoE, and 14.7% with apoCs as revealed by 3–15% gradient SDS-PAGE (Fig. 1). The labeled lipoproteins were kept under nitrogen and stored at 4°C for up to 1 week.

The specific activity of the lipoproteins ranged from 150 to 300 cpm/ng protein depending on the lipoprotein fraction used. Lipoprotein concentrations are expressed in terms of protein content.

Plasma lipoprotein turnover

Lipoprotein turnover studies were carried out after 14 days of drug administration. At 8 AM, the hamsters received an i.v. bolus injection of ^{125}I-labeled hamster LDL, Me-LDL, VLDL, or [^{3}H]retinyl ester-labeled hamster chylomicrons via the left jugular vein, while the animals were kept under light halothane anesthesia. When labeled VLDL or chylomicrons were injected, a first blood sample (150 µl) was taken from the right jugular vein 15 sec after the bolus injection for the determination of the amount of radioactivity in the plasma. When labeled LDL were injected, the first blood sample was taken up to 5 min after the injection. The amount of radioactivity circulating at these time points was set to be 100%. When indicated, hamsters received an injection of lactoferrin (70 mg/kg), 1 min prior to the injection of the radiolabeled lipoproteins. Blood samples were then collected from the right jugular vein at various time points as indicated under Results. For quantitation of radioactivity associated with VLDL and LDL, the trichloroacetic acid (TCA)-precipitable ^{125}I-radioactivity remaining in the plasma was measured in a γ-counter (Cobra Auto-Gamma, Packard). For chylomicrons, the ^{3}H-radioactivity present in the plasma was determined by liquid scintillation counting (Tri-Carb 2200CA.) For the LDL turnover studies, the plasma decay curves were analyzed by a non-linear, least-squares curve-fitting procedure (RS/1 from BBN Software Products Corporation) and the fractional catabolic rates (FCR) were calculated by the two-pool Matthews model (17).

To determine the effects of Ro 16-6532 and lovastatin on the VLDL-LDL cascade, ^{125}I-labeled VLDL was injected into hamsters and the amount of the ^{125}I-radioactivity associated with apoB-100 in VLDL and LDL was followed for 4 h. For this purpose, blood was collected via the jugular vein from the injected hamsters under light halothane anesthesia at selected time intervals. For lipoprotein isolation, 100 µl plasma was diluted to 570 µl with unlabeled plasma coming from a pool obtained from normal hamsters. In order to visualize the lipoprotein fractions, 10 µl of 0.1% Sudan Black was added to all plasma samples and the plasma was adjusted to a density of 1.21 g/ml with KBr and overlayered with 400 µl of 0.15 M NaCl/0.27 mM EDTA, pH 7.4 (11). During the whole procedure the samples were kept on ice. Lipoproteins were separated by ultracentrifugation as described above. The stained VLDL, LDL, and HDL bands were isolated by tube slicing and the totality of each lipoprotein fraction (125 µl) was subjected to polyacrylamide gel electrophoresis as previously described (18) with slight modifications. Briefly, one-dimensional SDS-PAGE was performed in a Vertical Electrophoresis Unit (LKB 2001) on 3–15% linear gradient slab gels (14 × 12 × 0.5 cm). Gels were prepared with a LKB Multiphor II Gradient Maker using a 3 M Tris-HCl buffer (pH 8.8) containing 0.1% SDS. The isolated lipoprotein fractions were mixed with an equal volume (125 µl) of sample buffer (75 mM Tris-HCl, pH 7.0, containing 5% SDS, 10% glycerol 0.5% DL-dithiothreitol, and 0.05% Bromophenol Blue), heated at 95°C for 5 min and the 250-µl samples were applied to the gel. The electrode buffer was 25 mM Tris, pH 8.3, 0.19 M glycin, 0.1% SDS, and 0.02% thiodiglycol. Electrophoresis was carried out at 25–30 mA for 16–18 h at a temperature of 10°C. The gels were stained with Coomassie Blue, and the label in the apoB zone was measured in a γ-counter.

Liver lipoprotein uptake

^{125}I-labeled VLDL was injected into the left jugular vein of hamsters and 100 µl blood was collected from the right jugular vein 15 sec thereafter for determination of the total injected dose of radioactivity in the plasma. The hamsters were killed by decapitation 3 min after the injection of the ^{125}I-labeled VLDL; maximal liver uptake was already reached at this time point (19). The livers were immediately perfused in situ with saline through the portal vein for 3 min to remove the residual blood. The whole organ was excised and weighed. As the distribution of the radioactivity is very homogeneous over the whole liver (data not shown), pieces of liver are representative for the total radioactivity taken up by the liver. Therefore, three tissue samples (0.8–1.0 g each) were taken from the three main lobes for measurement of the radioactivity (γ-counter). To determine the uptake of [^{3}H]retinyl ester-labeled chylomicrons in the liver, the same procedure was used except that the animals were killed 30 min after the injection of the labeled chylomicrons as label uptake in the liver is maximal at 30 min after the injection (19). Four liver samples weighing 150–200 mg each were solubilized in Soluene®-350 (Packard) prior to liquid scintillation counting. When indicated, hamsters received an injection of lactoferrin (70 mg/kg) 1 min prior to the injection of ^{125}I-labeled VLDL or [^{3}H]retinyl ester chylomicrons.

Ligand blotting procedures for LDL-receptor

Procedure 1 (as performed in Basel, Switzerland). For ligand blot assays with hamster ^{125}I-labeled VLDL and ^{125}I-labeled LDL, liver membranes from control and drug-treated hamsters were prepared as previously described (20) and the 8,000–100,000 *g* microsomes were solubilized by incubation with the nonionic detergent octyl-β-D-glucoside (21). The soluble microsomal extract was subjected to SDS-PAGE under non-reducing conditions and proteins were separated as previously described (22). Electrophoretic transfer of proteins to nitrocellulose membranes (Probind 45, LKB) was performed by horizontal semi-dry blotting (120 mA for 180 min) with a Multiphor II Novablot Electrophoretic Transfer Unit (LKB), as previously described (23). After a 30-min preincubation period in 10 mM Tris-HCl, 150 mM NaCL, 2 mM $CaCl_2$, 5% BSA, pH 8.0, at 37°C, the filters were incubated for 120 min at room temperature in the same buffer containing ^{125}I-labeled VLDL or ^{125}I-labeled LDL (15–20 µg of protein/ml). The nitrocellulose membrane was washed, dried, and exposed to Kodak XAR-5 film for 8–16 h at −70°C in a cassette containing two Cronex Lightning Plus intensifying screens (DuPont).

Procedure 2 (as performed in Huddinge, Sweden). For ligand blot assays with rabbit ^{125}I-labeled β-VLDL, liver membranes from control and drug-treated hamsters were prepared by a two-step procedure as described for human liver membranes (24). Membrane proteins were separated on SDS-PAGE (6%) (22) and electrotransferred onto nitrocellulose filters (BA 85, Schleicher & Schuell) with a Bio-Rad transblot cell (500 mA for 20 h). The filters were preincubated for 60 min at room temperature in 50 mM Tris-HCl, 2 mM $CaCl_2$, 5% BSA, pH 8.0. Rabbit ^{125}I-labeled β-VLDL was then added (5 µg of protein/ml) for an additional 60-min incubation period. The nitrocellulose membranes were washed, dried, and subjected to autoradiography (Cronex film with Quanta III intensifying screens (DuPont). For quantification, the LDL-receptor bands of the blots were cut out for γ-counting.

Determination of VLDL secretion rate

Triton WR 1339 is able to block the clearance of triglyceride-rich lipoproteins (VLDL) by inhibiting their lipolytic degradation (25, 26), thus allowing a measure of the rate of VLDL secretion by the increase of plasma triglycerides (27).

In preliminary experiments, an i.v. bolus injection of Triton WR 1339 (600 mg/kg body weight, using a solution of 30% (w/v) Triton in 0.9% NaCl) into control hamsters fed the standard chow diet or the coconut-supplemented chow diet was confirmed to result in linear increases in plasma triglyceride levels for at least 4 h, corresponding to a rapid accumulation of VLDL concomitant with a loss of LDL and HDL (**Fig. 2**). These results are in accordance with other published data (28), and in line with the hypothesis that in tritonized animals the newly secreted VLDL cannot be removed from the circulation or be converted to LDL (29).

This technique gives no information about the origin of the secreted triglycerides; however, high liver and low intestinal lipid secretion was achieved by fasting the animals overnight before Triton injection.

Analytical and biochemical procedures

In order to assess the distribution of cholesterol among the plasma lipoproteins in control and treated animals, 20-µl plasma aliquots were applied on a small-bore column containing Superose 6 gel (Smart™, Pharmacia) as described previously (30), and total cholesterol was measured in each 50-µl elution fraction. This chromatography technique (FPLC) results in excellent separation of VLDL from LDL, but a perfect base-line separation between LDL and HDL was not obtained, especially in plasma from fat-fed hamsters. The amount of cholesterol associated with LDL or HDL was therefore estimated by assuming a gaussian distribution for

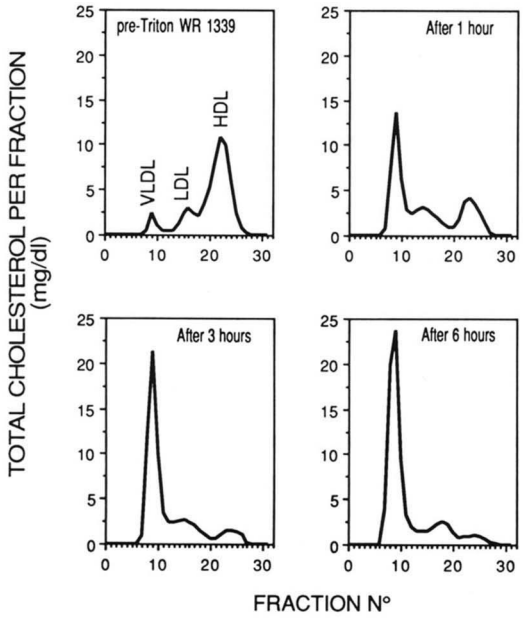

Fig. 2. Typical evolution of the cholesterol profile of plasma lipoproteins (VLDL, LDL, HDL) of control hamsters fed a normal chow diet after intravenous injection of Triton WR 1339 (600 mg/kg). Lipoprotein separations were performed by Superose 6 gel chromatography and cholesterol concentration was measured enzymatically in each elution fraction.

VLDL, LDL, and HDL, using a nonlinear, least-squares curve-fitting procedure (RS/1 from BBN Software Products Corporation) to calculate the area under the curves. The protein concentration was determined by the bicinchoninic method, (BCA™ Protein Reagent, Pierce, Rockford). The plasma concentrations of cholesterol and triglycerides were determined by enzymatic colorimetric procedures in 96-well microplates with the <Roche> Cholesterol PAP and the <Roche> Triglycerides PAP, (Roche Diagnostica, Kaiseraugst). For the measurements of small amounts of cholesterol in the lipoprotein fraction obtained by FPLC separation, a fluorescent method was used (31).

Determinations of hamster apoA-I and apoB-100 were done in a sandwich-format ELISA, using polyclonal IgG antibodies obtained from rabbits immunized with purified hamster apoA-I or apoB-100. The apoA-I/ELISA assay had a measurement range of 0–10 ng/ml, a detection limit of about 200 pg/ml, and an inter-assay CV of 4–5%. The apoB-100/ELISA assay had a measurement range of 0–50 ng/ml, a detection limit of about 1 ng/ml, and an inter-assay CV of 3–9%.

Statistical comparisons were done by the two-tailed Student's *t*-test for unpaired observations.

RESULTS

Effect of diet on plasma lipids, lipoprotein profiles, and apolipoprotein levels

The concentrations of plasma lipids in hamsters fed the standard diet were comparable to those reported by others for male hamsters (32). Feeding the coconut-supplemented or coconut-plus-cholesterol-supplemented diets to hamsters led to significant increases in all plasma lipid levels as compared to feeding the normal chow diet (**Fig. 3A; Table 2,** control groups). In the coconut-supplemented diet, free cholesterol and triglycerides increased by 54% and 40%, respectively, whereas both cholesteryl esters and phospholipids increased by 22%, similar to previously reported findings (33). A 3-fold increase of plasma free cholesterol and triglycerides and

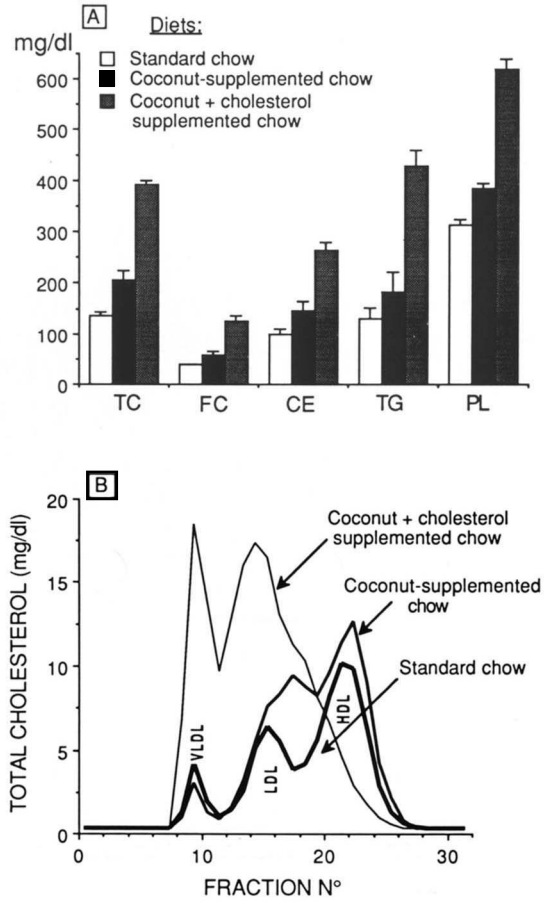

Fig. 3. Plasma lipid levels (A) and cholesterol profile of plasma lipoprotein (B) in control hamsters fed different diets. Upper panel: Male hamsters (n = 5/group) were fed for 3 weeks with 10 g per day of the different diets. Total cholesterol (TC), free cholesterol (FC), cholesteryl esters (CE), triglycerides (TG), and phospholipids (PL) were determined enzymatically. Each bar represents the mean ± SEM. Lower panel: Typical cholesterol profiles of plasma lipoprotein from hamsters fed different diets. Lipoprotein separations were performed by Superose 6 gel chromatography and cholesterol concentration was measured enzymatically in each elution fraction.

TABLE 2. Effects of Ro 16-6532 and lovastatin on plasma total cholesterol and triglycerides in hamsters fed with different diets

Experimental Groups	Plasma Total Cholesterol			Triglycerides		
	BT	AT	% Change[a]	BT	AT	% Change[a]
	mg/dl		%	*mg/dl*		%
Standard chow						
Control	149 ± 23	157 ± 18	+6	137 ± 36	136 ± 40	−1
Ro 16-6532 (1 mmol/kg)	143 ± 20	84 ± 20[b]	−42	141 ± 21	146 ± 58	+4
Lovastatin (25 μmol/kg)	142 ± 16	134 ± 9	−6	153 ± 34	176 ± 51	+15
Lovastatin (190 μmol/kg)	134 ± 19	97 ± 6[b]	−28	150 ± 31	412 ± 85[b]	+174
Coconut-supplemented diet						
Exp. 1						
Control	238 ± 36	228 ± 23	−4	164 ± 30	189 ± 50	+15
Ro 16-6532 (1 mmol/kg)	233 ± 20	120 ± 9[b]	−49	154 ± 45	166 ± 58	+8
Lovastatin (25 μmol/kg)	248 ± 23	165 ± 24[b]	−34	162 ± 41	247 ± 69	+52
Exp. 2						
Control	204 ± 14	204 ± 20	0	206 ± 51	170 ± 43	−18
Ro 16-6532 (300 μmol/kg)	203 ± 20	154 ± 9[b]	−24	155 ± 33	134 ± 17	−14
Ro 16-6532 (1 mmol/kg)	208 ± 7	108 ± 7[b]	−48	191 ± 29	174 ± 16	−9
Lovastatin (185 μmol/kg)[c]	215 ± 17	110 ± 8[b]	−49	166 ± 13	533 ± 90[b]	+222
Coconut + cholesterol-supplemented chow						
Control	345 ± 22	391 ± 18	+13	266 ± 55	439 ± 86	+65
Ro 16-6532 (1 mmol/kg)	451 ± 92	160 ± 14[b]	−65	244 ± 82	139 ± 30[b]	−43
Lovastatin (25 μmol/kg)	405 ± 80	340 ± 52	−16	236 ± 67	140 ± 68[b]	−41

Hamsters were treated as described in Materials and Methods. Results are expressed as mean ± SD for five animals per group, except lovastatin (185 μmol/kg). BT, day before first drug administration; AT, after 14 days of drug administration.

[a]Percent change from pretreatment.

[b]Significantly different from control absolute values ($P < 0.05$).

[c]Number = three animals.

a 2-fold increase of cholesteryl esters and phospholipids with respect to control chow were observed in hamsters fed a coconut-supplemented diet containing 0.12 wt% of added cholesterol, again in agreement with others (34).

Fig. 3B, and **Table 3** (control groups) show the effects of these diets on the distribution of cholesterol among plasma lipoproteins as assessed by FPLC. Feeding the animals with the coconut-supplemented diet produced a 94% increase of LDL-cholesterol and a 47% increase of HDL-cholesterol whereas VLDL-cholesterol remained unchanged. They also had a nearly 3-fold higher apoB-100 level, and a 23% increase of the apoA-I level.

By adding 0.12 wt% cholesterol to the coconut-supplemented diet the lipoprotein profile was extremely altered. VLDL-cholesterol increased by about 6-fold; LDL-cholesterol also increased whereas less cholesterol is eluted in the HDL-fraction. The latter may be caused

TABLE 3. Effects of Ro 16-6532 and lovastatin on lipoprotein levels in hamsters fed with a standard diet and a coconut-supplemented diet

	VLDL-C	LDL-C	HDL-C	ApoB-100	ApoA-I
	mg/dl			*mg/ml*	
Standard chow					
Control	22 ± 1	58 ± 5	127 ± 8	0.44 ± 0.06	1.21 ± 0.09
Ro 16-6532 (1 mmol/kg)	15 ± 3	11 ± 4[a]	62 ± 6[a]	0.25 ± 0.04[a]	0.79 ± 0.07[a]
Lovastatin (25 μmol/kg)	55 ± 14[a]	44 ± 8	97 ± 6[a]	0.78 ± 0.21	1.09 ± 0.05
Coconut-supplemented chow					
Control	19 ± 7	113 ± 26	187 ± 22	1.20 ± 0.23	1.49 ± 0.32
Ro 16-6532 (1 mmol/kg)	9 ± 1	46 ± 7[b]	122 ± 18	0.92 ± 0.02[b]	1.31 ± 0.10
Lovastatin (25 μmol/kg)	18 ± 4	64 ± 4	131 ± 8	1.26 ± 0.11	1.18 ± 0.12
Lovastatin (50 μmol/kg)	62 ± 28[b]	43 ± 3[b]	100 ± 19[b]	1.34 ± 0.20	1.05 ± 0.06[b]

Data are from plasma of hamsters treated for 14 days. The lipoprotein classes were isolated from individual animals by Superose 6 gel chromatography as described in Materials and Methods. Cholesterol was determined enzymatically and apolipoproteins B-100 and A-I were measured separately by ELISA using polyclonal antibodies raised against hamster apoB-100 and apoA-I. Values are expressed as mean ± SEM, n = 5.

[a]$P < 0.05$ versus control animals on normal diet.

[b]$P < 0.05$ versus control animals on fat diet.

by a decrease in small HDL as well as by a shift of the HDL particle size to larger diameters co-eluting with LDL upon FPLC separation (Fig. 3B).

Drug effects on plasma lipid levels

Administration of Ro 16-6532 in a dose of 1 mmol/kg per day caused a sustained decrease in total plasma cholesterol in hamsters fed standard rodent chow without or with coconut supplementation, but free of exogenous cholesterol. Both in the hamsters fed the standard chow diet (**Fig. 4A**) and in the hamsters fed the coconut-supplemented diet (Fig. 4B), Ro 16-6532 brought the plasma cholesterol level down to a steady-state value of around 100 mg/dl. The maximal effect of the drug was reached after 4 or 8 days of treatment, respectively, and was maintained for the rest of treatment duration. After termination of drug administration, plasma cholesterol levels returned to the pretreatment or control levels within 3 days.

The effects of Ro 16-6532 and lovastatin on plasma cholesterol and triglyceride levels in the three dietary conditions are presented comprehensively in Table 2.

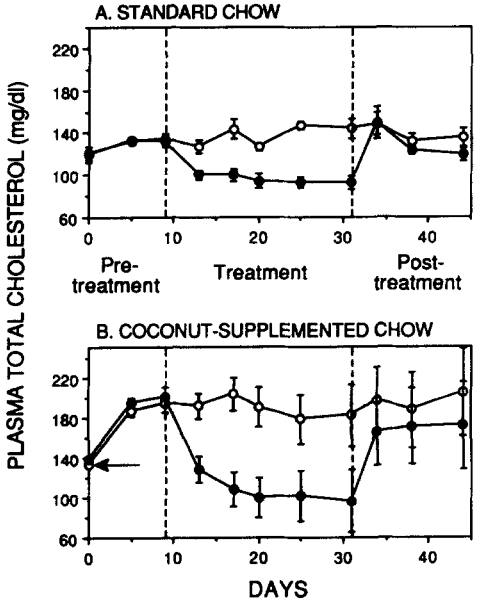

Fig. 4. Effect of Ro 16-6532 on total cholesterol concentration of plasma from male hamsters fed standard chow (A) and coconut-supplemented chow (B). One group of hamsters (n = 6) was kept on a standard chow diet. From day 10 until day 32 the animals received Ro 16-6532 mixed with the diet at a dose of 1 mmol/kg per day. A control group of untreated hamsters (n = 6) fed the standard chow was run in parallel. At the indicated times blood was collected, plasma was isolated, and total cholesterol concentration was determined (panel A). A second group of hamsters (n = 5) originally on a standard chow diet was switched (arrow) to a coconut-supplemented chow diet for 10 days. The animals received Ro 16-6532 (1 mmol/kg per day) from day 10 to day 32. A control group of untreated hamsters (n = 6) fed the coconut-supplemented chow was run in parallel; (○) untreated animals; (●) drug-treated animals. Each value represents the mean ± SEM.

Ro 16-6532 (1 mmol/kg per day for 14 days) reduced the plasma cholesterol level by 42% in hamsters fed the standard diet, and by 49% and 48% in two independent studies in hamsters fed the coconut-supplemented diet, and did not affect plasma triglycerides. With a dose of 0.3 mmol/kg per day a 24% reduction of the plasma cholesterol level was observed. When tested in the hamsters fed the coconut-supplemented diet containing 0.12 wt% cholesterol, Ro 16-6532 lowered plasma cholesterol by 65%, and plasma triglycerides by 43%.

In hamsters fed the standard diet lovastatin administration at a dose of 25 μmol/kg per day did not affect total plasma cholesterol and triglyceride levels; at a dose of 190 μmol/kg per day it lowered plasma cholesterol by 28% but increased plasma triglycerides 3-fold (Table 2). In hamsters fed the coconut-supplemented diet lovastatin at 25 μmol/kg per day reduced the plasma cholesterol level by 34% and tended to increase the triglyceride level. At 185 μmol/kg per day the plasma cholesterol level was lowered by 49%, while the plasma triglyceride level increased by 222%. In the coconut-plus-cholesterol-supplemented diet lovastatin at 25 μmol/kg per day caused a small (statistically nonsignificant) reduction of the cholesterol level (−16%), but a considerable decrease (−41%) of plasma triglycerides.

Effect of drugs on plasma lipoprotein profiles and apoB-100 and apoA-I levels

Addition of 0.12 wt% exogenous cholesterol to the coconut-supplemented diet strongly alters the composition and the size of the hamster plasma lipoproteins, rendering the evaluation of drug effects on LDL and HDL virtually impossible (Fig. 3B). The effects of the two drugs on the plasma lipoprotein profile were therefore not studied in the cholesterol-enriched diet.

As shown in Table 3, in hamsters fed the standard diet or the coconut-supplemented diet Ro 16-6532 caused a strong decrease of LDL-cholesterol (−80% and −60%, respectively), concomitant with a decrease of apoB-100 (−43% and −23%, respectively). However, the drug also caused a reduction of HDL-cholesterol (−52% and −35%, respectively) and apoA-I (−35% and −12%, respectively). VLDL-cholesterol was not significantly affected.

In hamsters on the standard chow, lovastatin in a dose of 25 μmol/kg per day lowered both LDL-cholesterol and HDL-cholesterol by 24%, but increased the VLDL-cholesterol concentration by 151% (accounting for the lack of change of total cholesterol noted above). In parallel the plasma concentration of apoB-100 tended to increase (by 77% but NS). In hamsters fed the coconut-supplemented diet, lovastatin at 25 and 50 μmol/kg per day caused dose-dependent decreases of LDL-cholesterol (−43% and −62%, respectively), of HDL-cholesterol (−30% and −47%, respectively) and of apoA-I

(–21% and –30%, respectively). In contrast, the higher dose led to a marked increase of VLDL-cholesterol (by 225%) and a slight increase of plasma apoB-100.

Effect of diets and drugs on plasma lipoprotein metabolism

The experiments described below were performed in order to find out how Ro 16-6532 or lovastatin are able to bring about the observed decrease of plasma LDL: do these drugs increase LDL removal from the plasma compartment, or/and do they decrease LDL production?

Turnover of hamster LDL. The FCR of LDL via the LDL-receptor pathway can be estimated as the difference between the FCR of labeled native LDL and that of Me-LDL in which the receptor recognition site in apoB has been blocked by chemical modification (12). ^{125}I-labeled LDL and Me-LDL from control animals were injected into control and drug-treated hamsters and the plasma decay rates of the lipoproteins were compared.

In hamsters on the coconut-supplemented diet, treatment with Ro 16-6532 or with lovastatin had no effect on the plasma clearance of either LDL or Me-LDL (**Fig. 5A, 5B**). As positive control the same labeled hamster LDL and Me-LDL were injected into male control rats and in rats treated with 17α-ethinyl estradiol. The reason for using rats instead of hamsters for the estradiol experiments was that hamsters do not up-regulate liver LDL-receptors in response to ethinyl estradiol treatment (35). As expected from previous studies (35–38) estradiol treatment led to a marked increase in the clearance of hamster LDL while it did not affect that of Me-LDL (Fig. 5C).

The FCR values of labeled LDL and Me-LDL in hamsters on the three diets are given in **Table 4.** The FCR of LDL via LDL-receptors was increased by 17% in animals fed the coconut-supplemented diet and decreased by 41% in animals fed the coconut-supplemented diet containing 0.12% cholesterol in comparison to hamsters fed the standard chow diet. The FCR of LDL via the LDL-receptor was not significantly affected by Ro 16-6532 in hamsters fed the standard chow and also not by Ro 16-6532 or lovastatin in hamsters on the coconut-supplemented chow. In hamsters fed the cholesterol-supplemented diet both Ro 16-6532 and lovastatin treatment completely prevented the decrease of the FCR caused by the addition of cholesterol to the diet.

Turnover and liver uptake of hamster VLDL. As the direct removal of LDL was not influenced by Ro 16-6532 and lovastatin in hamsters fed the standard or coconut-supplemented diets, the effect of these drugs on the metabolism of VLDL was investigated. **Figure 6** shows the fate of ^{125}I-labeled apolipoproteins in the plasma of

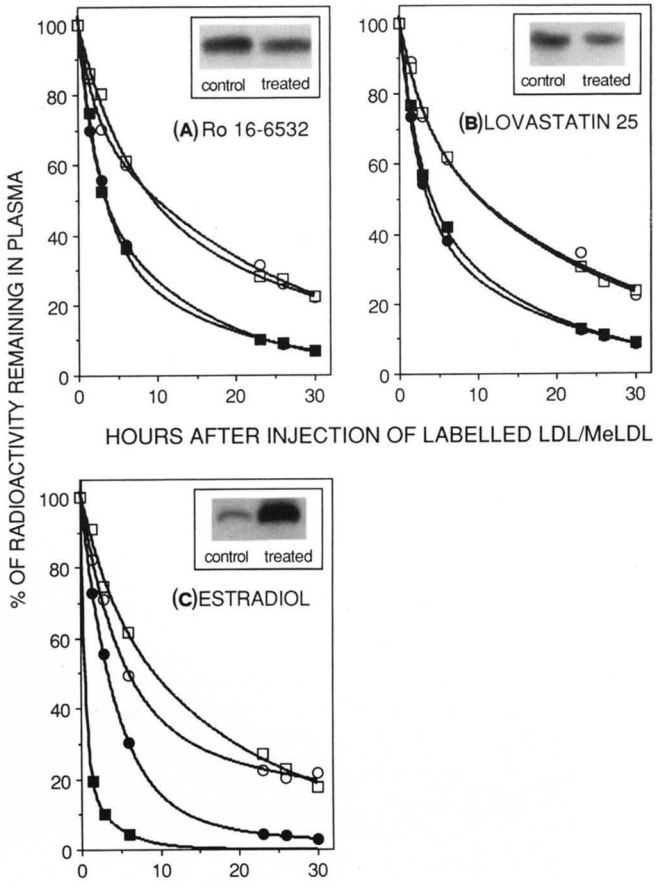

Fig. 5. Plasma clearance of labeled hamster LDL and labeled methylated hamster LDL in hamsters treated for 14 days with Ro 16-6532 (1 mmol/kg per day) (upper left panel) and lovastatin (25 μmol/kg per day) (upper right panel). Hamster ^{125}I-labeled LDL and methylated ^{125}I-labeled LDL (MeLDL) were injected intravenously (15 μg protein/animal) into drug-treated hamsters and control hamsters. As a positive control, hamster ^{125}I-labeled LDL and ^{125}I-labeled MeLDL were also injected into estradiol-treated rats (lower left panel). Blood was collected from the jugular vein at the indicated times and TCA-precipitable radioactivity was determined in 50-μl plasma samples. Hamster ^{125}I-labeled LDL (250 cpm/ng protein) injected into control animals (●) and drug-treated animals (■); hamster ^{125}I-labeled MeLDL (150 cpm/ng protein) injected into control animals (○) and drug-treated animals (□). Animals were fed the coconut-supplemented chow. Values are the mean from three animals per group. Inserts represent the ligand blots of LDL receptors in solubilized liver membranes from hamsters treated with Ro 16-6532, lovastatin and 17α-ethinyl estradiol (see procedure 1 in Materials and Methods). For this blotting experiment, 200 μg of solubilized protein per lane was electrophoresed. Hamster ^{125}I-labeled LDL concentration used as ligand was 3×10^6 cpm/ml (250 cpm/ng protein).

control and Ro 16-6532-treated hamsters on the coconut-diet, after injection of hamster ^{125}I-labeled VLDL. In contrast to labeled LDL, having a half-life time of about 10 h (Fig. 5), ^{125}I-labeled apoB-100 in VLDL has a half-life time of about 45 min in control hamsters (Fig. 6A). In the Ro 16-6532-treated animals the first phase of the clearance of VLDL particles is strongly accelerated,

TABLE 4. Fractional catabolic rate (FCR) of ^{125}I-labeled LDL and ^{125}I-labeled methylated LDL injected into hamsters fed different diets and treated with Ro 16-6532 (1 mmol/kg) or lovastatin (25 μmol/kg)

	Fractional Catabolic Rate		
	Standard Chow Diet	Coconut-Supplemented Chow Diet	Coconut-Supplemented Chow +0.12% Cholesterol Diet
		pools/day	
^{125}I-labeled LDL			
Control	2.30 ± 0.09[b,c]	2.80 ± 0.12	1.73 ± 0.10
Ro 16-6532	2.51 ± 0.23[c]	2.78 ± 0.03	3.27 ± 0.43[a]
Lovastatin		2.43 ± 0.21	2.87 ± 0.02[a]
^{125}I-labeled MeLDL			
Control	0.96 ± 0.05[b]	1.23 ± 0.05	0.94 ± 0.05
Ro 16-6532	1.49 ± 0.02[a,b]	1.16 ± 0.01	1.38 ± 0.17[a]
Lovastatin		1.24 ± 0.03	1.14 ± 0.06
Receptor-mediated[d]			
Control	1.34 ± 0.05[b,c]	1.57 ± 0.06	0.79 ± 0.05
Ro 16-6532	1.02 ± 0.20[b,c]	1.62 ± 0.02	1.89 ± 0.26[a]
Lovastatin		1.19 ± 0.18	1.73 ± 0.04[a]

Animals were fed the corresponding diet for 7 days (pretreatment), then fed the same diet containing Ro 16-6532 or lovastatin for 14 days. Homologous ^{125}I-labeled native LDL or methylated-LDL were injected (15 μg prot./100 μl) in control and drug-treated hamsters. Blood samples were obtained from jugular vein puncture at 5 min and then over 30 h. TCA-insoluble radioactivity remaining in each sample was measured and FCR was calculated as described in Materials and Methods. Values are means ± SEM from three animals per group.

[a]Significantly different from corresponding control ($P < 0.05$).

[b]Significantly different from corresponding treatment in coconut-supplemented diet ($P < 0.05$).

[c]Significantly different from corresponding treatment in coconut + cholesterol-supplemented diet ($P < 0.05$).

[d]FCR of native hamster LDL minus that of methylated-LDL.

the half-life of ^{125}I-labeled apoB-100 in VLDL being reduced to about 3 min (Fig. 6A). At the same time, the appearance of ^{125}I-labeled apoB-100 in the circulating LDL fraction was reduced in the Ro 16-6532-treated animals (Fig. 6B). However, in the period between 2 and 4 h after injection of labeled VLDL, the plasma clearances of ^{125}I-labeled apoB-100 in the LDL fraction of control and Ro 16-6532-treated animals are parallel. This confirms the lack of an effect of Ro 16-6532 on LDL turnover as observed after direct injection of labeled hamster LDL.

As can be seen by comparing Fig. 6A and 6B, only about 30% of the amount of labeled VLDL-apoB appears in the LDL-fraction on control hamsters. The balance is irreversibly lost from the circulation. In the animals treated with Ro 16-6532, the capacity for this

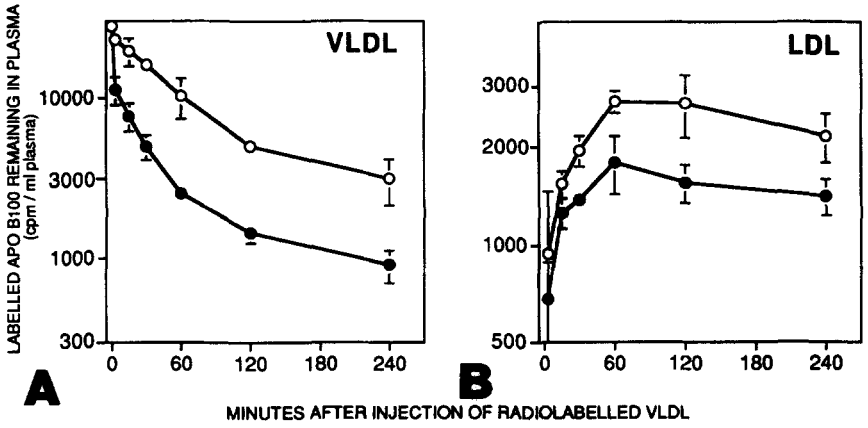

Fig. 6. Plasma clearance of labeled hamster VLDL in hamsters treated with Ro 16-6532. Hamster ^{125}I-labeled VLDL were injected intravenously into drug-treated hamsters (●) and control hamsters (○). At the indicated times blood samples were taken from the jugular vein. Plasma lipoproteins were isolated by density gradient ultracentrifugation and the distribution of radiolabeled apoB-100 in VLDL and LDL fractions was determined after protein separation by 3–15% gradient SDS-PAGE. Animals were fed the coconut-supplemented chow containing Ro 16-6532 (1 mmol/kg per day) for 14 days. Each value represents the mean ± SEM of data obtained in three animals.

TABLE 5. Effect of Ro 16-6532 and lovastatin on plasma decay and liver uptake of VLDL and chylomicrons in hamsters with and without prior injection of lactoferrin

	% of Injected Radioactivity			
	No Lactoferrin		After Lactoferrin[a]	
	Plasma	Liver	Plasma	Liver
	%		%	
^{125}I-labeled VLDL				
Control	86.6 ± 7.7	9.4 ± 0.7	101.8 ± 6.0	4.2 ± 0.5
Ro 16-6532	51.0 ± 3.8[b]	31.6 ± 6.7[b]	98.9 ± 6.2	6.2 ± 1.1
Lovastatin	81.5 ± 7.4	15.4 ± 0.8[c]	104.7 ± 8.2	4.0 ± 0.6
[^{3}H]retinyl ester chylomicrons				
Control	32.7 ± 7.6	31.2 ± 2.6	54.9 ± 5.1	15.3 ± 3.6
Ro 16-6532	23.2 ± 3.8	44.5 ± 1.1[c]	55.0 ± 1.8	15.3 ± 2.2
Lovastatin	36.5 ± 5.4	31.2 ± 5.6	49.6 ± 0.1	16.7 ± 3.8

Hamster ^{125}I-labeled VLDL and hamster [^{3}H]retinyl ester chylomicrons were injected intravenously into drug-treated hamsters and control hamsters. Three minutes after the injection of labeled VLDL or 30 min after the injection of labeled chylomicrons blood was collected by jugular vein puncture and the animals were killed by decapitation. The livers were perfused in situ with saline through the portal vein for 3 min. The whole liver was removed and radioactivity was measured as described in Materials and Methods. Prior to injection of labeled lipoproteins, animals were fed the coconut-supplemented chow and treated for 14 days with Ro 16-6532 (1 mmol/kg/day) and lovastatin (25 μmol/kg/day). Each value represents the mean ± SEM of data obtained in three animals.

[a]Intravenous injection of lactoferrin (70 mg/kg), 1 min prior to the injection of the labeled VLDL and chylomicrons.

[b]Significantly different from corresponding control ($P < 0.01$).

[c]($P < 0.05$).

irreversible removal is apparently increased, so that even less LDL is formed. In subsequent experiments we set out to establish which tissues were responsible for this increased VLDL removal during the first clearance phase. As shown in **Table 5,** in control animals 3 min after injection of ^{125}I-labeled VLDL, about 85% of the injected radioactivity was found in the plasma, while the liver contained about 10% of the injected dose. Uptake into the intestinal wall was about 0.1% from the injected radioactivity and about 2% was recovered from all other organs (data not shown). In Ro 16-6532-treated animals plasma clearance and liver uptake of labeled VLDL were 3-fold higher as compared to control animals. In lovastatin-treated hamsters plasma removal and liver uptake were also increased albeit to a lesser extent (Table 5).

Turnover and liver uptake of hamster chylomicrons. [^{3}H]retinyl ester-labeled chylomicrons from donor hamsters were injected into recipient control and drug-treated hamsters. As [^{3}H]retinyl ester is a stable chylomicron core marker, the clearance of chylomicron remnants can be determined by following the disappearance of plasma radioactivity (14). As shown in **Fig. 7,** 65% of the injected dose is removed from the plasma of control animals within 30 min after injection. In the Ro 16-6532-treated hamsters this clearance rate was increased such that 75% of the injected ^{3}H was removed from the plasma within 30 min. The chylomicron clearance rate was unchanged in the lovastatin-treated group. As for VLDL, the liver is the most important organ for chylomicron remnant clearance. We found 31% of the injected radioactivity in the liver of control animals 30 min after injection of [^{3}H]retinyl-labeled ester chylomicrons (Table 5). This percentage was enhanced to 45%

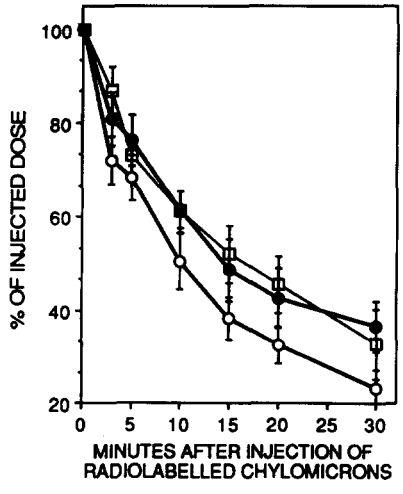

Fig. 7. Plasma clearance of labeled chylomicrons from the plasma in hamsters treated for 14 days with Ro 16-6532 (1 mmol/kg per day) and lovastatin (25 μmol/kg per day). Hamster chylomicrons containing 3.6×10^5 dpm of [^{3}H]retinyl ester were injected intravenously into drug-treated hamsters and control hamsters. Blood was collected from the jugular vein at the indicated times and plasma was assayed for radioactivity. Animals were fed the coconut-supplemented chow. Each value represents the mean ± SEM of data obtained in four animals; (□), controls; (○), Ro 16-6532-treated; (●), lovastatin-treated.

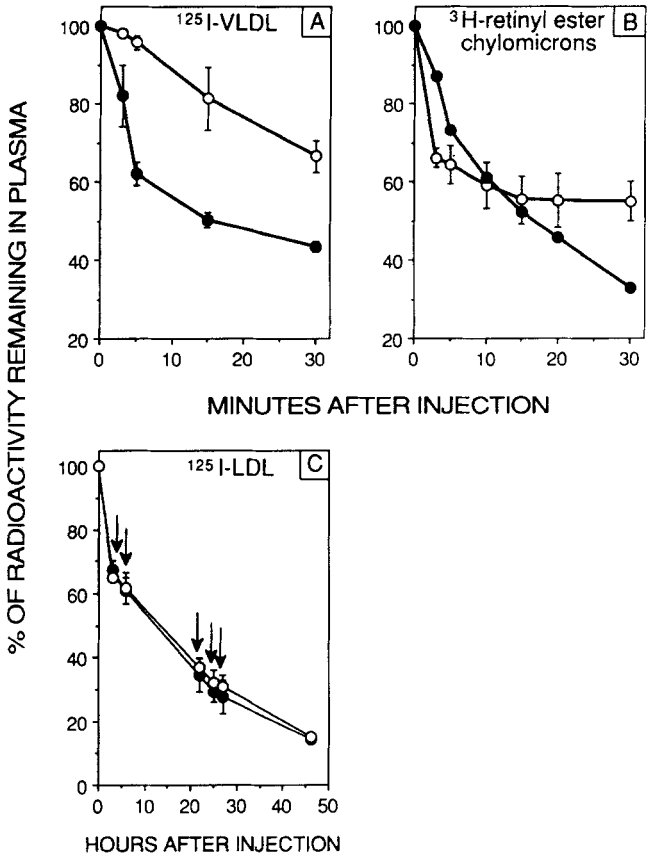

Fig. 8. Effect of lactoferrin on plasma clearance of ^{125}I-labeled VLDL (A), [^{3}H]retinyl ester chylomicron (B), and ^{125}I-labeled LDL (C) in control hamsters fed a coconut-supplemented chow. The labeled lipoproteins were injected into hamsters and blood was collected from the jugular vein at the indicated times and plasma was assayed for radioactivity (●). Some hamsters received an intravenous injection of lactoferrin (70 mg/kg), 1 min prior to the injection of the labeled lipoproteins (○). In panel C, one group of animals (○) received a first intravenous injection of lactoferrin 1 min prior to the injection of labeled LDL and then repetitive injections of lactoferrin (indicate by arrows), 1 min before each blood collection. Each value represents the mean ± SEM of data obtained in three animals.

in Ro 16-6532-treated hamsters, whereas it was not changed in lovastatin-treated animals (Table 5).

Effect of lactoferrin injection on lipoprotein metabolism and liver uptake

Other authors (19, 39, 40) have shown that preinjection of lactoferrin before injection of VLDL or chylomicron leads to a prolonged residence time of their remnants in the plasma as a consequence of a blockade of the liver uptake. We could confirm this for the hamster: lactoferrin clearly retarded the initial phase of clearance of labeled VLDL (**Fig. 8A**) and the late phase of the clearance of chylomicrons (Fig. 8B) from the plasma, and decreased the net uptake of these labels in the liver (Table 5). In contrast, lactoferrin, even given in multiple injections, has no effect on the clearance rate of ^{125}I-labeled LDL in hamsters (Fig. 8C). In separate experiments it was found that lactoferrin could not compete with ^{125}I-labeled LDL for binding to the hepatic LDL-receptor, and that ^{125}I-labeled lactoferrin itself could not bind to the liver LDL-receptor (blots not shown). Of interest for the present study, the preinjection of lactoferrin completely annihilated the increase in plasma clearance and liver uptake of labeled VLDL and chylomicrons induced by Ro 16-6532 or by lovastatin (Table 5, columns 3 and 4).

Effect of drug treatment on amount of lipoprotein receptor protein in liver membranes

The experiments described above showed that neither Ro 16-6532 nor lovastatin affected the turnover of LDL, whereas both drugs caused an increased turnover and liver uptake of VLDL. We then set out to study the effect of these drugs on the amount of functional lipoprotein binding proteins extracted from liver membranes, as measured in ligand blotting experiments. We observed a clear decrease of LDL-binding protein in liver membranes of hamsters fed the cholesterol-supplemented diet as compared to the hamsters on the chow-diet and coconut-supplemented diet (data not shown), in agreement with the LDL-turnover studies presented in Table 4, thus validating this methodology.

Treatment of hamsters on the coconut-supplemented diet with Ro 16-6532 or with 25 μmol/kg per day lovastatin did not affect or even tended to decrease the amount of LDL-binding protein in liver membranes (Fig. 5, inserts), again in harmony with the kinetic data given in Table 4. As a positive control, treatment of rats with ethinyl estradiol clearly increased the binding of labeled LDL to liver membranes (Fig. 5C, insert).

The lack of stimulation and the tendency for a reduction of LDL-binding protein expression in liver membranes by the two drugs was confirmed independently in the Huddinge laboratory, using ^{125}I-labeled rabbit β-VLDL as a high-affinity probe for the LDL-receptor (24) (**Fig. 9**). No other proteins were detected in the liver membrane extracts of the control and the drug-treated hamsters which were able to bind this labeled lipoprotein.

Effect of diet and drugs on VLDL secretion

In order to validate that Triton WR-1339 blocks VLDL catabolism and clearance from the plasma, ^{125}I-labeled VLDL was injected into hamsters given a bolus injection of Triton WR-1339 5 min earlier. As shown in **Fig. 10,** 1 h after injection of ^{125}I-labeled VLDL in tritonized hamsters, 100% of the injected dose still was recovered

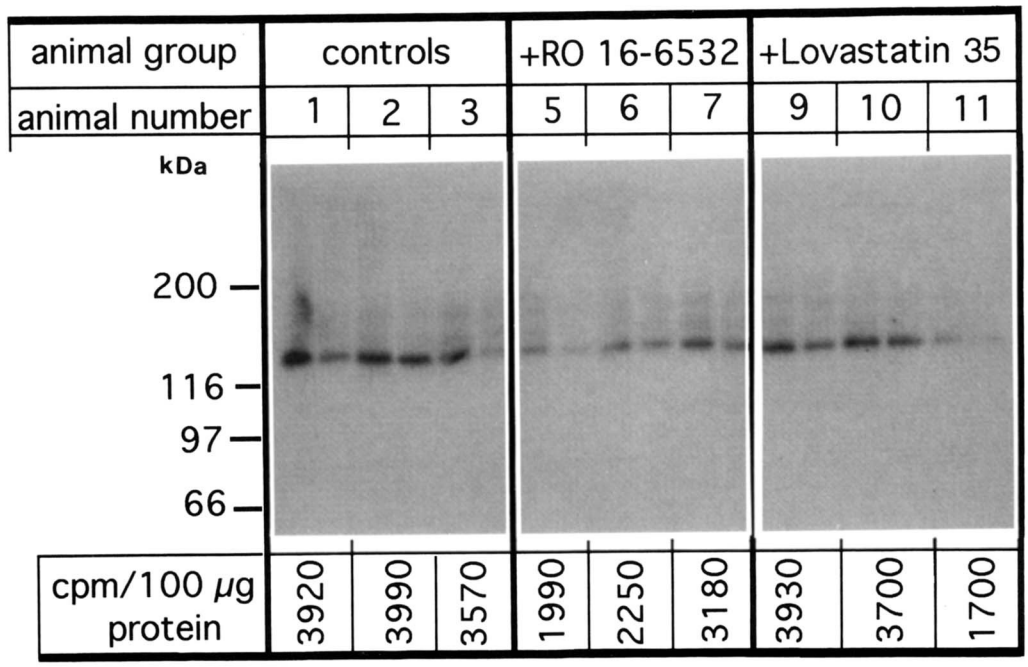

Fig. 9. Ligand blots of liver membranes from hamsters fed the standard chow and treated for 14 days with Ro 16-6532 (1 mmol/kg per day) and lovastatin (35 μmol/kg per day). Liver membranes from three drug-treated hamsters and three control hamsters were prepared and separated on SDS-PAGE as described in Materials and Methods, procedure 2. For each individual, two lanes were loaded with 100 μg and 200 μg of membrane protein, respectively. The separated proteins were transferred to a nitrocellulose membrane and incubated with rabbit ^{125}I-labeled βVLDL (5 μg of protein/ml). After wash the membrane was exposed onto X-ray film for 3 h. The cpm-values were obtained by counting of γ-radioactivity of excised 130 kDa band corresponding to the LDL-receptor. Data are averages from two lanes normalized per 100 μg of protein after subtraction of background filter activity that averaged 870 cpm.

in the plasma and only about 20% of radioactivity was removed after 4 h.

As shown in **Table 6,** in hamsters on the standard diet triglyceride levels increased linearly for at least 6 h, reaching a 12-fold higher level above basal. Treatment with Ro 16-6532 or lovastatin had no significant effect on this increase in triglycerides compared with the control group.

In animals fed the coconut-supplemented diet the basal triglyceride level was higher (220 mg/dl); triton injection again induced a 12-fold increase of the triglyceride value (to 2500 mg/dl) within 6 h. Apparently, the VLDL-secretion rate is elevated by coconut-fat supplementation. The triton-induced increase of plasma triglyceride levels was significantly reduced by 28% ($P < 0.05$) in animals treated with Ro 16-6532 compared with the control group, whereas lovastatin treatment had no effect.

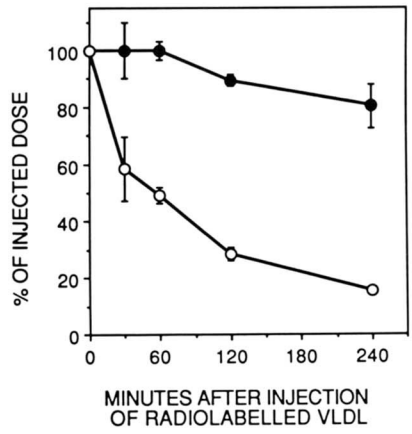

Fig. 10. Effect of Triton WR-1339 on the plasma clearance of VLDL in hamsters. Hamster ^{125}I-labeled VLDL (8 μg protein, 170 cpm/ng) was injected intravenously into control hamsters fed a standard chow (○). One group of animals received a bolus injection of Triton WR 1339 (600 mg/kg body weight) prior to the injection of ^{125}I-labeled VLDL (●). Blood was collected from the jugular vein at the indicated times and plasma was assayed for radioactivity. Values are the mean ± SEM from three animals per group.

DISCUSSION

Over the last decade the male golden Syrian hamster has become a popular model for the study of lipid and

TABLE 6. Changes in concentrations of plasma triglycerides after Triton WR 1339 injection in hamsters fed a standard diet and a coconut-supplemented diet and treated with Ro 16-6532 (1 mmol/kg/day) and lovastatin (25 μmol/kg/day) for 14 days

	Triglycerides (Time after Triton WR 1339 Injection)						
	0 min	30 min	60 min	120 min	180 min	240 min	360 min
	mg/dl						
Standard chow							
Control	121 ± 19	266 ± 28	397 ± 19	649 ± 41	876 ± 34	1176 ± 30	1453 ± 56
Change	0	145 ± 15	275 ± 14	528 ± 38	754 ± 44	1055 ± 41	1332 ± 70
Ro 16-6532	108 ± 16	204 ± 29	331 ± 32	538 ± 55	822 ± 65	1065 ± 83	1410 ± 67
Change	0	97 ± 20	223 ± 25	430 ± 46	714 ± 53	957 ± 75	1303 ± 63
Lovastatin 25	129 ± 25	258 ± 32	353 ± 42	560 ± 47	785 ± 72	1050 ± 91	1366 ± 126
Change	0	129 ± 19	224 ± 24	431 ± 36	657 ± 49	922 ± 70	1237 ± 101
Coconut-supplemented chow							
Control	216 ± 44	476 ± 78	676 ± 96	1085 ± 200	1406 ± 194	1849 ± 297	2477 ± 35
Change	0	260 ± 50	461 ± 65	869 ± 163	1190 ± 152	1633 ± 253	2261 ± 311
Ro 16-6532	173 ± 15	397 ± 18	556 ± 32	832 ± 79	1142 ± 98	1379 ± 122	1798 ± 144[a]
Change	0	223 ± 13	382 ± 23	658 ± 75	969 ± 92	1205 ± 121	1624 ± 147[a]
Lovastatin 25	480 ± 154	758 ± 134	998 ± 149	1299 ± 123	1806 ± 164	2127 ± 177	2892 ± 235
Change	0	278 ± 45	518 ± 60	819 ± 81	1326 ± 141	1647 ± 239	2412 ± 281

Each value represents the mean ± SEM of data obtained in five animals.
[a]$P < 0.05$ versus control at 360 min.

lipoprotein metabolism (28, 32–34, 41–49), and we selected this animal for our studies for reasons mentioned by the cited authors.

Effect of addition of coconut fat or coconut fat plus cholesterol to the diet

As the standard chow diet corresponds to the usual diet of these laboratory animals, and the coconut-supplemented diet reflects the present-day Western diet in humans with respect to its macronutrient composition, these two diets were primarily used for our pharmacological studies. These diets contained a low amount (6 mg per 100 g dry weight) of cholesterol, exposing our hamsters to a cholesterol dose of 0.6 mg per day (about 4.5 mg/kg per day). Although this cholesterol dose is not far removed from the dose received by humans eating a Western diet (~ 500 mg/day, or ~ 7 mg/kg per day), we also used a diet with 0.12 wt% added cholesterol (leading to a dose of 12 mg/day, or about 85 mg/kg per day). As demonstrated by the elegant studies performed in the Dietschy laboratory (42–45), the latter diet represents a setting in which cholesterol absorption is of major importance in determining hepatic cholesterol homeostasis and lipoprotein metabolism in the hamster.

Although it was not the primary goal of this study to characterize the effects of addition of fat and cholesterol to the diet on hamster lipoprotein metabolism, the data collected in these investigations (summarized in **Table 7**) still deserve some comments. The following conclusions can be drawn.

1. The rise in plasma levels of cholesterol, triglycerides, LDL-cholesterol and apoB-100 in hamsters caused by coconut supplementation is in line with many other studies (34, 43, 47–49). Of these, Woollett, Spady, and Dietschy (49) were the first to study the effect of saturated fats against the background of a chow diet without any added cholesterol. They found that the addition of coconut-oil had little effect on the rate of LDL-clearance, but increased the plasma LDL level due to a higher rate of LDL production. In partial agreement with this study, we even observed a small increase of the FCR of LDL via the LDL-receptor upon coconut supplementation, and report for the first time an increased VLDL production (Table 6).

2. Adding extra cholesterol to the coconut-diet led to a further rise of plasma cholesterol and triglycerides, as has been described in previous hamster studies (42, 43, 47–49). Initially, a decreased LDL-receptor activity was

TABLE 7. Summary: effects of feeding the coconut-supplemented or coconut-plus-cholesterol-supplemented diets as compared to the chow diet

	Coconut	Coconut plus Cholesterol
Pla-chol	↑	↑↑
Pla-TG	↑	↑↑
VLDL-chol	→	↑↑
LDL-chol	↑	↑↑
HDL-chol	↑	↓
ApoB	↑	-
ApoA-I	→/↑	-
LDL-FCR	↑	↓
LDL-receptor	→	↓
VLDL secretion	↑	-

↑: Increase above control; ↓: decrease above control; ↑↑: increases above control and above coconut alone; →: no effect with respect to control; -: not done.

invoked to explain these changes (43, 48–51), and we also found a decreased FCR of LDL and a decreased amount of hepatic LDL-receptors as compared to the supplementation with coconut only. However, in more recent studies, cholesterol addition to the diet was found to stimulate hepatic secretion of apoB-100-containing lipoproteins both in the absence and presence of extra dietary triglycerides (49, 52–55). We feel that this mechanism makes a greater contribution to the increase of plasma cholesterol level, because in the cholesterol-fed hamsters lovastatin clearly increased the FCR of LDL (Table 4) but nevertheless had only little effect on the total plasma cholesterol level (Table 2).

3. The increase of HDL and apoA-I upon coconut-supplementation of the diet in hamsters confirm the findings of others (56–58) with respect to dietary saturated fats in other animal species. Hayek et al. (58) were able to ascribe this increase to both a rise of the production rates and a decreased FCR of HDL cholesterol esters and apoA-I.

Effects of Ro 16-6532 or lovastatin on lipoprotein levels and metabolism in hamsters on different diets

The effects of the two drugs observed in our experiments with hamsters on three different diets are summarized in **Table 8.** The following conclusions can be drawn.

1. In hamsters on the coconut-supplemented diet the decrease of LDL concentration caused by Ro 16-6532 and lovastatin (at the 25 μmol/kg per day dose) can be ascribed to an increase of the plasma clearance and liver uptake of VLDL, and a reduced conversion of VLDL into LDL. In addition, Ro 16-6532 treatment led to a decrease of the VLDL secretion rate in these animals.

Although we did not study VLDL turnover in chow-fed hamsters, the drop of LDL and apoB-100 caused by Ro 16-6532 is likely to be caused by enhanced hepatic VLDL clearance as well, as neither VLDL secretion (Table 6) nor LDL turnover (Table 4) were affected in this condition.

The increased hepatic uptake of labeled VLDL apparently is mediated via a receptor different than the LDL-receptor. This must be inferred not only from the lack of up-regulation of the LDL-clearance or amount of LDL-receptors in the liver by the drugs, but also from the finding that pre-injection of lactoferrin, which does not affect LDL-clearance (Fig. 8), completely blocked the drug-induced up-regulation of VLDL and chylomicron (-remnant) clearance (Table 5). (We have no explanation for the difference in kinetics of the inhibitory effect of lactoferrin on the VLDL vs. chylomicron clearance, as seen in Fig. 8.)

It is worth stressing that treatment with Ro 16-6532 in the hamsters on chow diet or coconut-supplemented diet did not affect plasma triglyceride level. This indicates that the increased plasma clearance and hepatic uptake of VLDL is not due to an increased rate of lipolytic processing,[3] but rather to an increased capacity of the liver to capture VLDL at the remnant stage. The kinetics of the VLDL-clearance display a distinct biphasic pattern (Fig. 6A, 8A), which we ascribe to heterogeneity of the labeled VLDL population containing both "young" and "old" particles. The latter, being close to the remnant stage, are those that are cleared first after re-injection into acceptor animals. As is clear from Fig. 6A, only the clearance of these "old" particles is enhanced in the animals treated with Ro 16-6532, while the clearance of the younger particles was not affected (presumably because the rate of their clearance is lim-

[3]Activities of lipoprotein lipase and hepatic lipase in plasma and liver of drug-treated animals also were not different than those in controls, Dr. H. Jansen, unpublished observations.

TABLE 8. Summary: effects of Ro 16-6532 or lovastatin as compared to no drug controls, in hamsters on the three different diets

	Standard Diet		Coconut-Suppl.		Coconut + Cholesterol	
	Ro 16-6532	Lovastatin	Ro 16-6532	Lovastatin	Ro 16-6532	Lovastatin
Pla-chol	↓	↓	↓	↓	↓	→
Pla-TG	→	↑	→	↑	↓	↓
VLDL-chol	→	↑	→	↑	-	-
LDL-chol	↓	↓	↓	↓	-	-
HDL-chol	↓	↓	→	↓	-	-
ApoB	↓	→	↓	→	-	-
ApoA-I	↓	→	→	↓	-	-
LDL-clearance	→	-	→	→	↑	↑
LDL-receptor	-	-	→	→	-	-
VLDL-clearance	-	-	↑	↑	-	-
VLDL secretion	→	→	↓	→	-	-

↑: Increase above control; ↓: decrease above control; ↑↑: increases above control and above coconut alone; →: no effect with respect to control; -: not done.

ited by the rate of lipolytic processing, which is slower than terminal hepatic uptake). This also explains why the drug effects on chylomicron clearance were much smaller (Ro 16-6532) or absent (lovastatin) than those on VLDL-clearance (Fig. 7): the labeled chylomicrons are obtained by lymph cannulation, and therefore consist of a more homogeneous population of "young" particles.

2. The decrease of plasma cholesterol obtained with Ro 16-6532 in hamsters on the cholesterol-supplemented diet was bigger than the decrease in hamsters on the other two diets. In these animals, the drug caused an increase of the LDL-clearance relative to the no-drug controls, which may have contributed to the fall of plasma cholesterol. However, lovastatin had a similar-sized stimulatory effect on the receptor-mediated LDL-clearance in this condition (Table 4), but nevertheless was unable to significantly reduce the plasma cholesterol level (Table 2). We conclude that the drop of plasma cholesterol caused by Ro 16-6532 in the cholesterol-fed hamsters again was not mediated by up-regulation of LDL-receptors. Instead, the drug largely prevents the absorption of the added cholesterol (8), and so prevents the rise in hepatic cholesterol and VLDL secretion which (as discussed above) is at least partly responsible for the increase of plasma cholesterol induced by cholesterol feeding. This also would explain why lovastatin, being not a (good) inhibitor of cholesterol absorption, has less effect on plasma cholesterol in this dietary condition.

3. It is generally believed that statins lower LDL levels by increasing the hepatic LDL uptake through up-regulation of LDL-receptors. The latter has certainly been documented in some animal studies (59–61) and in humans (62). However, recent investigations have revealed a variety of other explanations for the LDL reduction on statin therapy, both in humans (63–68) as well as in different animal species (69–71).

That lovastatin can cause a drop in LDL production was inferred already by Grundy and Vega (63) and Vega, Krauss, and Grundy (64), which they ascribed to enhanced clearance of VLDL remnants through the LDL-receptor. However, more recently, simvastatin was also shown to increase chylomicron remnant removal (66), the clearance of which is not considered to be mediated by LDL-receptors. Several authors describe a change in the LDL (68, 69) or VLDL (70, 71) particle composition induced by statin treatment, leading to better receptor binding and more rapid plasma clearance of these particles without requiring increased expression of LDL receptors. However, this was excluded by us in separate experiments in which we injected radiolabeled LDL from control or drug-treated hamsters into control hamsters, and observed the same clearance rates of these two tracers (data not shown).

Statins were also found to directly inhibit VLDL production rates (65, 72) or the cholesterol content of the newly secreted VLDL particles (73). This we also could not confirm in our experiments (Table 6). Finally, still others found that statins inhibit the direct production of LDL-apoB (68, 74). So far, there is only one report describing the effect of a statin on lipoprotein metabolism in the hamster (60). Therein the authors describe a rise of LDL-receptor mRNA in the liver with lovastatin added to the diet (normal chow) at levels of 0.05% or more (equivalent to about 100 μmol/kg body weight per day), but not at lower doses which yet were able to lower plasma cholesterol. In our experiments, lovastatin (25 μmol/kg per day) did not affect (even tended to decrease; Table 4) the FCR of LDL via the LDL-receptor or the rate of VLDL secretion in hamsters on the chow or coconut-supplemented diets.

The explanation that we can offer for the decrease of LDL cholesterol and apoB-100 by this dose of lovastatin is up-regulation of VLDL remnant clearance by a hepatic uptake mechanism different than the LDL receptor, as elaborated in point 1, above. The molecular identity of the components of this uptake machine is still unknown; an important characteristic is that it recognizes lactoferrin as well as VLDL remnants. Potential candidates for this remnant uptake machine are *a)* LDL-receptor related protein (75), *b)* heparan sulfate proteoglycans (76), *c)* lipoprotein lipase (77), or *d)* the lipolysis-stimulated receptor (78). Future work must clarify whether one or more of these entities is up-regulated by the two drugs studied.

4. Lovastatin causes a remarkable increase of plasma triglycerides and of VLDL-cholesterol and a tendency to increase apoB-100 in the hamsters fed the chow or the coconut-supplemented diet. Similar findings were reported previously by Amin, Gustafson, and Perrone (79). The reason for this increase is unclear. It does not seem to be caused by an increased VLDL secretion, as there was no effect of lovastatin in the Triton experiment. One possibility is that lovastatin inhibits the amount or activity of lipoprotein lipase, which also would explain why the plasma levels of HDL and apoA-I are decreased by lovastatin in these hamsters.

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