Hypocholesterolemic actions of atorvastatin are associated with alterations on hepatic cholesterol metabolism and lipoprotein composition in the guinea pig

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Abstract. Guinea pigs were fed 15% (w/w) fat, high in lauric and myristic acids, a diet known to produce hypercholesterolemia in these animals. The diet was given alone or in combination with four doses of atorvastatin equivalent to 1, 3, 10, and 20 mg/kg per day. Atorvastatin reduced plasma LDL cholesterol concentrations by 46, 50, 53, and 70%, respectively ($P < 0.001$). Plasma apoB concentrations were reduced by atorvastatin ($P < 0.001$) and compositional changes occurred in VLDL and LDL with reductions of the relative proportion of cholesteryl ester and increases in triacylglycerol. A reduction in hepatic cholesteryl ester (66%) was observed only with the highest atorvastatin dose (20 mg/kg per day) while microsomal cholesterol was reduced by 30% with 3–20 mg/kg per day. Hepatic ACAT activity was down-regulated and apoB/E receptor number was increased by atorvastatin. In contrast, HMG-CoA reductase activity and cholesterol 7α-hydroxylase were not affected by the drug. VLDL apoB secretion rates were decreased by atorvastatin treatment 59 and 76% with 3 and 20 mg/kg per day, respectively. Nascent VLDL particles were larger after drug treatment, showing an increased number in triacylglycerol molecules. These results support the hypothesis that the plasma LDL lowering induced by atorvastatin is due to a decreased secretion of apoB in combination with an increase of hepatic apoB/E receptors.—**Conde, K., M. Vergara-Jimenez, B. R. Krause, R. S. Newton, and M. L. Fernandez.** Hypocholesterolemic actions of atorvastatin are associated with alterations on hepatic cholesterol metabolism and lipoprotein composition in the guinea pig. *J. Lipid Res.* 1996. **37:** 2372–2382.

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Coronary heart disease is associated with high mortality and morbidity in Western societies and especially in the United States. Atherosclerosis is a disease that continues to kill more than 500,000 Americans annually despite the greater success in treatment (1). Atherosclerosis and coronary heart disease are related to high levels of cholesterol in plasma.

Hypercholesterolemic patients considered to be at high risk for developing coronary heart disease are the main target for drug therapy. The use of a group of compounds known as HMG-CoA reductase inhibitors for lowering plasma cholesterol levels has increased in recent years (2, 3). Several studies have been conducted to address the effects and mechanisms of action of these drugs (4–8).

Atorvastatin (CI-981) is a chiral, calcium salt of a pentasubstituted pyrrole (9) that has been tested in humans and animal models (9–12). Atorvastatin treatment in hypercholesterolemic patients has been shown to produce a dose-dependent reduction of total plasma cholesterol as well as LDL cholesterol (9).

Atorvastatin has also been studied for its effects on atherosclerosis progression or regression, alone or combined with other inhibitors (10). Studies in casein-fed rabbits treated with different doses of atorvastatin have shown a dose-dependent reduction of total plasma cholesterol levels (11). Results from several studies suggest that the mechanism of action of this compound is mainly based on a decreased production of apoB-containing lipoproteins (12, 13).

Studies conducted until now have addressed different

Abbreviations: HMG-CoA, 3-hydroxy-3-metylglutaryl coenzyme A; VLDL, very low density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein; RID, radioimmunodiffusion; ACAT, acyl-CoA:cholesterol acyltransferase; TAG, triacylglycerol; FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; CETP, cholesteryl ester transfer protein.

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aspects of HMG-CoA reductase inhibitors and their role in plasma cholesterol reduction. There are still many questions regarding the mechanisms of action of these compounds. Some studies suggest that atorvastatin reduces cholesterol in plasma by decreasing hepatic secretion of apoB-rich lipoproteins without having an effect on the number of receptors (11, 12). The present study was conducted to test the effects of atorvastatin on cholesterol and lipoprotein metabolism in guinea pigs. Hepatic cholesterol homeostasis and its relation to lipoprotein secretion was studied in detail by measuring the activity of the regulatory enzymes of cholesterol metabolism. Guinea pigs were used as the animal model because of similarities to humans, LDL:HDL ratio, distribution of cholesterol pools (free cholesterol being the major pool in liver), activities of main enzymes regulating cholesterol metabolism, and most important, because guinea pigs, similar to humans, respond to dietary and drug treatments by changing plasma LDL cholesterol concentrations (8).

MATERIALS AND METHODS

Materials

Cholesterol oxidase, cholesterol esterase, peroxidase, and cholesterol kits were purchased from Boehringer Mannheim (Indianapolis, IN). Phospholipid and free cholesterol kits were obtained from Wako (Osaka, Japan). Triacylglycerol kit was obtained from Sigma (St. Louis, MO). ^{125}I was purchased from New England Nuclear Research Products (Boston, MA); halothane from Halocarbon (Hackensack, NJ); Beckman cellulose propionate tubes from Beckman (Palo Alto, CA), DL-hydroxy-[3-^{14}C]methyl glutaryl coenzyme A (1.81 GBq/mmol) and DL-[5-^{3}H]mevalonic acid (370 Gbq/mmol) were purchased from New England Nuclear (Boston, MA). Glucose-6-phosphate, glucose-6 phosphate dehydrogenase, Tyloxapol (Triton WR-1339), and NADP were obtained from Sigma (St. Louis, MO). Oleoyl-[1-^{14}C]coenzyme A (1.8 GBq/nmol) was obtained from Amersham (Clearbrook, IL). Aquasol and liquiflor were from New England Nuclear (Boston, MA). Atorvastatin (lot # XH020193) was provided by the Parke-Davis, Research Division, Warner-Lambert Company.

Diets

Diets were prepared and pelleted by Research Diets, Inc. (New Brunswick, NJ). Isocaloric diets were designed to cover the guinea pig nutritional requirements. All diets had equal composition except for the amount of atorvastatin. Atorvastatin concentration in the different diets was: 0.0%, 0.005%, 0.017%, 0.05%, and 0.1%, corresponding to 0, 1, 3, 10, and 20 mg/kg per day. The amount of cholesterol in the diet was adjusted to be 0.04%; this amount is equivalent to 112 mg/1000 kcal or less than 300 mg/day for a human diet (14). The fat mix was olive oil–palm kernel oil–safflower oil (1:2:1.8), diets rich in lauric and mirystic acids known to cause endogenous hypercholesterolemia in guinea pigs. Fatty acid composition of the diet was 25% C12:0, and C14:0, 25% other saturated fatty acids, 25% C18:1, and 25% C18:2.

Animals

Male Hartley guinea pigs weighing 350–400 g were purchased from Sasco Inc. (Omaha, NE). Animals were randomly assigned to one of five different diets for 3 weeks. Three animals were placed per metal cage and they were kept in a light-cycle room (light from 7:00 to 19:00 h). Diet and water were provided ad libitum. Last exposure of the animals to the drug was just a few minutes before obtaining plasma samples. All animal experiments were conducted in accordance with U.S. Public Health Service/U.S. Department of Agriculture guidelines. Experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee.

Lipoprotein isolation

Blood was obtained by heart puncture from guinea pigs under halothane anesthesia. Plasma samples were collected and a preservation cocktail was added to the samples (aprotonin 0.5 ml/100 ml, PMSF 0.1 ml/100 ml and sodium azide 0.1 ml/100 ml). One ml of plasma from each animal was stored at 4°C for further analysis and the rest was used for lipoprotein isolation.

Lipoprotein isolation was done by sequential ultracentrifugation (15) in an L8-M ultracentrifuge (Beckman Instruments, Palo Alto, CA). Very low density lipoprotein (VLDL) was isolated at a density of 1.006 g/ml at 125,000 *g* at 15°C for 19 h in a Ti-50 rotor. Low density lipoprotein (LDL) was isolated in a density range of 1.019–1.09 g/ml in quick-seal tubes at 15°C for 18 h at 125,000 *g* (16). LDL samples were dialyzed in 0.09% NaCl–0.01% EDTA, pH 7.2, for 24 h and stored at 4°C for further analysis.

Plasma and liver lipids

Plasma samples were analyzed for cholesterol and triacylglycerols using enzymatic methods (17) and were also used for HDL cholesterol determination by precipitation with dextran sulfate (18).

Livers excised from guinea pigs after exsanguination were stored at −20°C for lipid analysis. Lipid extraction was done by the method of Carr, Andersen, and Rudel (19). Briefly, 1 g of liver was cut into small pieces and

combined with 10 ml chloroform–methanol 2:1 overnight. Lipid extraction was done by mixing with acidified water, and separating the two phases with a separatory funnel. An aliquot of 0.2 ml, taken from the lower phase, was evaporated to dryness and resuspended in 0.2 ml ethanol for enzymatic determination of total and unesterified cholesterol.

Hepatic microsome lipids

Free cholesterol was assayed in hepatic microsomes isolated from guinea pigs fed the different diets. Microsomes (2–3 mg protein) were treated with 20 volumes of chloroform–methanol 2:1 according to Folch, Lees, and Sloane Stanley (20). Samples were dried under nitrogen and lipids were solubilized with 1 ml of water with Triton 100X (1%). Free cholesterol and phospholipids were determined by enzymatic methods.

Determination of apoB concentration

Apolipoprotein B polyclonal antibodies were obtained by injection of guinea pig-purified LDL into a sheep. Antibodies were purified by affinity column chromatography. ApoB concentration was determined for plasma samples using the radioimmunodiffusion (RID) assay (21) in which the antigen was allowed to diffuse radially into wells containing the antibody. Agarose gel (0.2%) was used to precoat glass plates. Guinea pig LDL antiserum was incorporated in an agarose solution (1%) and cast on the precoated glass plate. Samples (15 µl) were loaded in wells made by cutting with a 4-mm gel punch (Bio-Rad). LDL from guinea pig isolated by affinity chromatography was used as standard. Plates were incubated at 37°C for 72 h. After removal of nonspecific proteins, gels were stained with Coomassie blue. Diameters of the immunoprecipitate rings were measured using a RID reader. Linear regression was used to calculate apoB concentrations in samples (21).

Lipoprotein characterization

VLDL and LDL were analyzed for phospholipids, triacylglycerols, free cholesterol, and total cholesterol by enzymatic methods (17). Esterified cholesterol was calculated as the difference between total cholesterol and free cholesterol. Protein was measured by the modified method of Lowry (22). LDL number of molecules were calculated based on one apolipoprotein B per LDL (apoB molecular mass 412,000 kD). The molecular weights were: 885.4, 386.6, 646, and 734 for triacylglycerol, free and esterified cholesterol, and phospholipids, respectively. LDL diameters were calculated according to Van Heek and Zilversmit (23).

Hepatic microsome isolation

Microsome isolation was done by the method described by Fernandez et al. (24). Briefly, livers obtained from guinea pigs on the different diets were pressed through a tissue grinder, placed in cold buffer (50 mmol/L KH_2PO_4, 0.1 mol/L sucrose, 50 mmol/L KCl. 50 mmol/L NaCl, 30 mmol/L EDTA, and 2 µmol/L dithiothreitol, pH 7.2), and homogenized with a Potter-Elvehjem homogenizer. The microsomal fraction was obtained after two centrifugations at 10,000 *g* for 15 min (JA-20 rotor in a J2-21 centrifuge, Beckman Instruments), and 1 h centrifugation at 100,000 *g* at 4°C. Samples were further homogenized and centrifuged for 1 additional hour at 100,000 *g* at 4°C. Microsomal pellets were resuspended in buffer, homogenized, and stored at −70°C for enzyme analysis. The protein content in the microsomes was measured by the method reported by Markwell et al. (22).

LDL binding

Hepatic microsomes were isolated as described above from guinea pigs from the control group and drug groups. LDL binding to hepatic microsomes was determined according to the method reported by Fernandez and McNamara (25). Briefly, pooled guinea pig LDL (from the control group and from the different treatment diets) was radiodinated by the iodine monochloride method of Goldstein, Basu and Brown (26) to give a specific activity of 150–400 cpm/ng. Hepatic microsomes, (200 µg of protein) were incubated in buffer B (NaCl, 100 mmol/L; $CaCl_2$, 0.5 mmol/L; Tris-HCl, 50 mmol/L; 20 mg/ml bovine serum albumin, pH 7.5) with different concentrations of ^{125}I-labeled LDL (10–80 µg/ml), with or without an excess of unlabeled human LDL (1 mg/ml) for 2 h at 37°C. Human LDL was used as a competitor for the determination of receptor-mediated binding (27). After incubation, 75 µl of incubation mixture was overlayered with 100 µl of 3% BSA buffer B for further centrifugation at 38,000 rpm for 45 min in a Ti-42.2 rotor. The supernatant was removed by aspiration and the pelleted microsomes were washed with 150 µl 3% BAS buffer B and recentrifuged for additional 30 min at 38,000 rpm at 4°C. The supernatant was removed, the tubes were sliced at the bottom, and the pellets were counted for radioactivity in a gamma counter (LKB-Wallace CliniGamma, Gaithersburg, MD). K_d and B_{max} were determined from Woolf plots (28).

HMG-CoA reductase assay

The activity of HMG-CoA reductase (E.C. 1.1.1.34) was measured in hepatic microsomes according to Shapiro, Imblum, and Rodwell (29). Microsomes were incubated with 7.5 nmol (0.33 Gbq/nmol) [3-^{14}C]HMG-CoA, 4.5 µmol glucose-6-phosphate, 3.6 µmol EDTA, 0.45 µmol NADP, 0.3 IU glucose-6-phosphate dehydrogenase, and 0.024 Gbq [^{3}H]mevalonic acid added as a recovery standard.

The reaction was stopped after 15 min with 10 M HCl (0.025 ml per assay) with an excess of mevalonic acid. Samples were incubated for another 30 min at 37°C to allow for the conversion of mevalonic acid to mevalonalactone. After incubation, microsomes were pelleted by centrifugation for 1 min at 1,000 *g*. An aliquot of the supernatant (0.1 ml) was applied to silica gel TLC plates and developed with acetone–benzene 1:1(v/v), and the area containing the mevalonate (R_f = 0.6–0.9) was scraped and mixed with 5 ml aquasol. Radioactivity was measured using a liquid scintillation counter (LSC). HMG-CoA activity is expressed as pmol of [^{14}C]mevalonate produced per min per mg microsomal protein. Recoveries of [^{3}H]mevalonate were 60–70%.

ACAT activity

Hepatic ACAT (E.C. 2.3.1.26) activity was measured by the incorporation of [^{14}C]oleoyl-CoA into cholesteryl ester in hepatic microsomes isolated from the five groups of animals according to Smith et al. (30). No exogenous cholesterol was added. Hepatic microsomes (0.8–1.0 mg protein per assay) were preincubated with albumin (84 mg/ml) and buffer (50 mmol/L KH_2PO_4, 0.1 mol/L sucrose, 50 mmol/L KCl, 30 mmol/L EDTA, and 50 mmol/L NaF) to a final volume of 0.18 ml for 5 min at 37°C. Five hundred μmol/L oleoyl-[1-^{14}C]coenzyme A (0.15 Gbq/pmol) was added and the samples were incubated for 15 min at 37°C. The reaction was stopped with 2.5 ml of chloroform–methanol 2:1, and [^{3}H]cholesteryl oleate (0.045 GBq per assay) was added as a recovery standard. Additional 2.5 ml of chloroform–methanol and 1 ml of acidified water (0.05% H_2SO_4) were added to the samples. Samples were mixed and allowed to stand overnight. The aqueous phase was removed and the samples were dried under nitrogen. Samples were resuspended in 0.150 ml of chloroform containing 30 μg unlabeled cholesteryl oleate. Samples were applied to silica gel TLC plates and developed with hexane–diethyl ether 9:1 (v/v). Cholesteryl oleate was visualized with iodine vapors, scraped from the plate, and radioactivity was counted. Recoveries of [^{3}H]cholesteryl oleate were between 70–90%.

Cholesterol 7α-hydroxylase

Cholesteryl 7α-hydroxylase (EC 1.14.13.7) activity was assayed according to the method modified by Jelinik et al. (31). [^{14}C]cholesterol was used as a substrate and delivered as cholesterol–phosphatidylcholine liposomes (1:8 by weight). After preparation by sonication, an NADPH-regenerating system (glucose-6-phosphate dehydrogenase, NADP, and glucose-6-phosphate) was included in the assay as a source of NADPH. After addition of glucose-6-phosphate dehydrogenase (0.3 I.U.), samples were incubated for an additional 30 min. The reaction was stopped by addition of 5 ml of chloroform–methanol 3:1 and 1 ml acidified water (5% sulfuric acid). Tubes were mixed, the top layer was discarded, and samples were dried under nitrogen. Samples and 7α- and 7β-hydroxycholesterol standards each were dissolved in 100 μl of chloroform, applied to silica gel TLC plates, and developed with ethyl acetate–toluene 3:2. The plate was placed on XAR-5 film with intensifying screen overnight and placed in iodine vapors to mark the 7α- and 7β-hydroxycholesterol standards. Using the film as a guide, the location of the [^{14}C]7α-hydroxycholesterol spots was determined, scraped from the plate, and counted in a liquid scintillation counter.

In vivo VLDL-TAG and apoB secretion rates

Animals were fasted 12 h prior to the experiment and during the 8 h of study to avoid interference from postprandial lipoproteins. VLDL catabolism was blocked with Triton WR-1339 (32) by injection through a catheter inserted in the internal carotid artery. Triton is a detergent used for the determination of VLDL-triacylglycerol (TAG) secretion rates as it blocks the clearance of this lipoprotein by interfering with the action of lipoprotein lipase (32). Triton WR-1339 was diluted to a final concentration of 20% with 0.9% NaCl and injected into guinea pigs at a dose of 100 mg/kg body weight. After injection, plasma samples were collected into EDTA-containing tubes at 0, 5, 10, 15, 20, 35, 50, 75, 120, 180, 300, and 480 min post-injection and used for analysis of plasma triacylglycerols by enzymatic methods. At the end of the study (480 min) animals were anesthetized with halothane vapors and exsanguinated by heart puncture. Nascent VLDL was isolated at a density of 1.006 g/ml with ultracentrifugation and dialyzed against 0.09% NaCl. Phospholipids, triacylglycerols, cholesterol, and protein were determined on nascent VLDL as described above for mature VLDL. Nascent VLDL numbers of molecules were calculated as described above for LDL.

VLDL apoB secretion rates were calculated by multiplying VLDL-TAG secretion rates × apoB concentration (%) divided by VLDL-TAG (%). ApoB protein concentration was determined by isopropanol precipitation of VLDL (33). Total protein and protein from the supernatant were measured by the modified Lowry procedure (22). ApoB concentration was obtained as the difference between total protein and protein in the supernatant.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered statistically significant. The Newman-Keuls test was used as post-hoc analysis. Data are presented as means ± stan-

TABLE 1. Plasma lipids and lipoprotein cholesterol distribution in guinea pigs treated with diets containing 0, 1, 3, 10, and 20 mg/kg atorvastatin

Diet	Plasma Lipids			Lipoprotein Cholesterol		
	TPC ($P = 0.0001$)	TAG	ApoB ($P = 0.0001$)	VLDL	LDL ($P = 0.0001$)	HDL
	mg/dL				*mg/dL*	
Control	94 ± 25^a	99 ± 42	70 ± 23^a	2 ± 1	84 ± 25^a	7 ± 1
AT, 1 mg/kg	57 ± 11^b	88 ± 21	42 ± 18^b	2 ± 1	45 ± 12^b	10 ± 5
AT, 3 mg/kg	53 ± 7^b	127 ± 40	36 ± 8^b	2 ± 1	42 ± 7^b	9 ± 1
AT, 10 mg/kg	50 ± 11^b	98 ± 20	34 ± 7^b	2 ± 1	39 ± 11^b	9 ± 3
AT, 20 mg/kg	35 ± 9^b	101 ± 24	26 ± 9^b	1 ± 0	26 ± 8^b	7 ± 2

Data are presented as mean ± SD; n = 6 per dietary group; n = 11 for control group. Values in the same column with different superscripts are significantly different (one-way ANOVA). Abbreviations: TPC, total plasma cholesterol; TAG, triacylglycerol; apoB, apolipoprotein B; AT, atorvastatin.

dard deviation. Linear regression was used to identify significant correlations.

RESULTS

Effects of atorvastatin on plasma lipids and lipoproteins

There was no difference in weight gain for guinea pigs fed the different diets either by day or during the entire feeding period, indicating that guinea pigs consumed comparable amounts of the diet (data not shown). After feeding the animals the experimental diets for 3 weeks, blood was isolated and plasma was analyzed for cholesterol and triacylglycerol concentrations. Atorvastatin had a clear effect on total plasma and LDL cholesterol (**Table 1**), with no effect on plasma triacylglycerols (Table 1). Atorvastatin reduced plasma total cholesterol in a dose-dependent manner, and this was due entirely to decreases in the LDL fraction. LDL cholesterol was reduced by 46%, 50%, 53%, and 70% for 1, 3, 10, and 20 mg/kg per day of atorvastatin respectively ($P < 0.001$). VLDL and HDL cholesterol were not affected by atorvastatin at the different doses tested (Table 1).

Plasma apolipoprotein B (apoB) concentration was decreased in a dose-dependent manner with atorvastatin treatment accounting for 40%, 48%, 51%, and 70% reduction for 1, 3, 10, and 20 mg/kg per day of atorvastatin respectively (Table 1). A strong correlation was found between apoB concentration and total plasma cholesterol ($r = 0.99$, **Fig. 1**). Such correlation indicates a lesser number of apoB-containing lipoproteins in animals receiving the drug treatment which may be due to a decreased production or increased clearance of these particles.

Effects of atorvastatin on VLDL and LDL composition

The composition of VLDL isolated from guinea pigs treated with atorvastatin was significantly altered (**Table 2**). Free cholesterol (FC) increased with 3 and 10 mg/kg per day of atorvastatin compared to the control group. An important change in VLDL composition was an 80% decrease in cholesteryl esters as a result of atorvastatin treatment (1.0 ± 0.9 vs. 0.1 ± 0.1) ($P = 0.02$).

LDL composition (%) was significantly altered with intake of 20 mg/kg per day of atorvastatin (**Table 3**). LDL cholesteryl ester (CE) was decreased by 29% (55 ± 6 vs. 39 ± 5) ($P = 0.002$). Triacylglycerols (TAG) ($P = 0.0001$) and phospholipids (PL) ($P = 0.005$) were increased by 175% (7 ± 2 vs. 18 ± 7) and 87% (8 ± 5 vs. 14 ± 1), respectively. The number of molecules of LDL components (CE, TAG, FC, and PL) were unaffected by atorvastatin treatment (data not shown). LDL

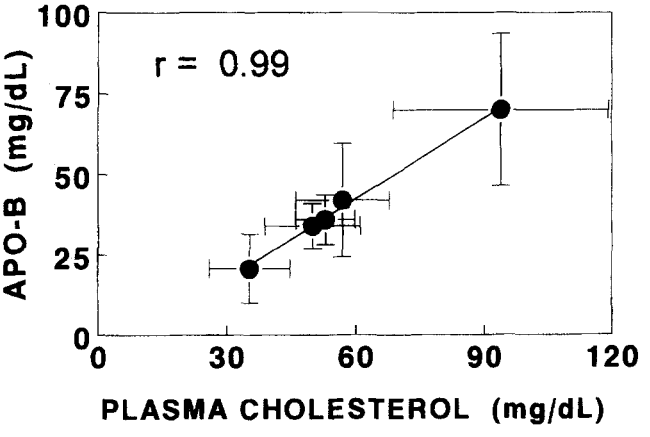

Fig. 1. Correlation between plasma cholesterol (mg/dL) and apoB (mg/dL) ($r = 0.99$, $P < 0.001$) of guinea pigs fed diets containing 20, 10, 3, 1, and 0 mg/kg of atorvastatin. Values represent the mean ± SD of n = 11 determinations for control animals, and n = 6 for animals treated with the different doses of atorvastatin.

TABLE 2. VLDL composition in guinea pigs treated with 0, 1, 3, 10, and 20 mg/kg atorvastatin

Diet	VLDL Composition				
	FC	CE ($P = 0.02$)	TAG	PL	PRO
	mg/dL				
Control	3 ± 1^b	1.0 ± 0.9^a	73 ± 4	13 ± 3^b	9 ± 2
AT, 1 mg/kg	3 ± 1^b	0.2 ± 0.4^b	70 ± 3	18 ± 4^a	9 ± 3
AT, 3 mg/kg	6 ± 1^a	0.1 ± 0.1^b	73 ± 2	11 ± 1^b	10 ± 2
AT, 10 mg/kg	5 ± 1^a	0.1 ± 0.1^b	75 ± 1	10 ± 2^b	10 ± 1
AT, 20 mg/kg	3 ± 0^b	0.1 ± 0.2^b	74 ± 2	13 ± 1^b	10 ± 2

Data are presented as mean ± SD; n = 6 per dietary group; n = 11 for control group. Values in the same column with different superscripts are significantly different; $P < 0.05$ (one-way ANOVA). Abbreviations: FC, free cholesterol; CE, cholesteryl ester; TAG, triacylglycerol; PL, phospholipids, PRO, protein; AT, atorvastatin.

TABLE 3. LDL composition in guinea pigs treated with 0, 1, 3, 10, and 20 mg/kg atorvastatin

Diet	LDL Composition					
	FC	CE ($P = 0.002$)	TAG ($P = 0.0001$)	PL ($P = 0.005$)	PRO	Diameter ($P = 0.04$)
	%					
Control	2 ± 1	55 ± 6^a	7 ± 2^b	8 ± 5^b	29 ± 3	330 ± 62^a
AT, 1 mg/kg	1 ± 1	53 ± 4^a	6 ± 1^b	9 ± 4^b	33 ± 2	303 ± 45^a
AT, 3 mg/kg	1 ± 0	55 ± 2^a	8 ± 1^b	7 ± 1^b	29 ± 2	337 ± 20^a
AT, 10 mg/kg	2 ± 1	51 ± 8^a	10 ± 3^b	9 ± 3^b	29 ± 1	322 ± 71^a
AT, 20 mg/kg	3 ± 0	39 ± 5^b	18 ± 7^a	14 ± 1^a	27 ± 2	261 ± 16^b

Data are presented as mean ± SD; n = 6 per dietary group; n = 11 for control group. Values in the same column with different superscripts are significantly different; $P < 0.04$ (one-way ANOVA). Abbreviations: FC, free cholesterol; CE, cholesteryl ester; TAG, triacylglycerol; PL, phospholipids; PRO, protein; AT, atorvastatin.

TABLE 4. Hepatic cholesterol and microsomal lipids concentrations of guinea pigs treated with 0, 1, 3, 10, and 20 mg/kg atorvastatin

Diet	Hepatic Cholesterol		Microsomal Lipids	
	Free	Esterified ($P = 0.001$)	FC ($P = 0.01$)	PL ($P = 0.006$)
	mg/g		*nmol/mg*	
Control	1.2 ± 0.4	0.26 ± 0.18^a	47 ± 12^a	351 ± 102^a
AT, 1 mg/kg	1.2 ± 0.2	0.17 ± 0.10^a	42 ± 3^a	374 ± 71^a
AT, 3 mg/kg	1.4 ± 0.2	0.16 ± 0.09^a	33 ± 8^b	367 ± 92^a
AT, 10 mg/kg	1.3 ± 0.2	0.15 ± 0.08^a	33 ± 7^b	205 ± 65^b
AT, 20 mg/kg	0.9 ± 0.1	0.13 ± 0.03^b	27 ± 6^b	316 ± 47^b

Data are presented as mean ± SD; n = 6 per dietary group; n = 11 for control group. Values in the same column with different superscripts are significantly different; $P < 0.01$ (one-way ANOVA). Abbreviations: FC, free cholesterol; PL, phospholipids; AT, atorvastatin.

isolated from guinea pigs treated with 20 mg/kg per day of atorvastatin exhibited a smaller diameter compared to the control group or the other atorvastatin groups (Table 3). These data indicate that atorvastatin treatment (at 20 mg/kg per day) yielded smaller triacylglycerol-rich LDL particles. Compositional changes in VLDL and LDL can affect the affinity of the apoB/E receptor for these particles.

Effects of atorvastatin on hepatic cholesterol

Hepatic cholesteryl ester was significantly decreased in guinea pigs treated with atorvastatin (20 mg/kg per day) (**Table 4**). There was no effect of this atorvastatin dose on either hepatic total or free cholesterol. No significant effects on hepatic cholesterol (free or esterified) were found with the other atorvastatin doses.

Effects of atorvastatin on hepatic microsomal lipids

Hepatic ACAT activity is regulated by the concentration of free cholesterol in the media. Because no correlation was found between hepatic cholesterol and ACAT activity, hepatic microsomal cholesterol was measured. Hepatic microsomal free cholesterol was reduced by 30% (47 ± 12 vs. 27 ± 6) with 3, 10, and 20

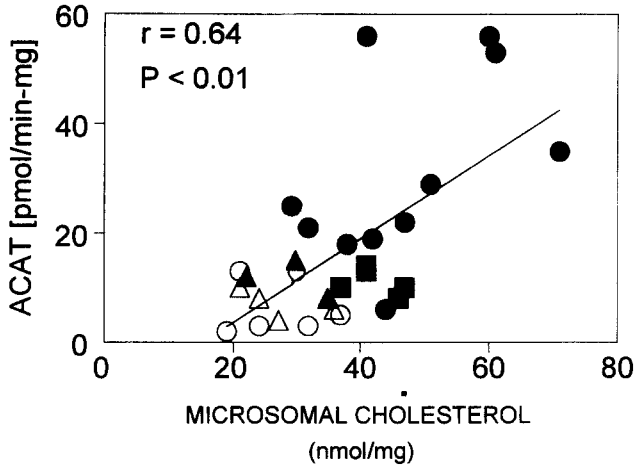

Fig. 2. Correlation between ACAT activity (pmol/min-mg) and microsomal free cholesterol (nmol/mg-protein) of guinea pigs fed diets containing 0 (●), 1 (■), 3 (△), 10 (▲), and 20 (○) mg/kg of atorvastatin (r = 0.64).

mg/kg per day of atorvastatin. Microsomal phospholipids were decreased by 10% (351 ± 102 vs. 316 ± 47) with 10 and 20 mg/kg per day of atorvastatin (Table 4). There was a strong correlation between ACAT activity and microsomal free cholesterol (r = 0.64) (**Fig. 2**). This correlation agrees with ACAT activity regulation by microsomal cholesterol. There was no significant effect on the phospholipid/free cholesterol ratio, indicating no change on hepatic microsomal membrane permeability in guinea pigs fed with atorvastatin.

Effects of atorvastatin on hepatic apo B/E receptor number

In order to test the effect of atorvastatin on LDL uptake by the liver, the number of hepatic apoB/E receptors was measured in hepatic microsomes. ApoB/E receptor number (B_{max}) increased by 29% (2.28 ± 0.63 vs. 2.95 ± 0.78) in microsomes isolated from animals treated with atorvastatin compared to the control group (**Table 5**). These results suggest that an increase in the number of receptors contributed to the decrease in LDL cholesterol. The dissociation constant (K_d) was not affected by the intake of atorvastatin (Table 5).

TABLE 5. LDL binding constants of hepatic microsomes of guinea pigs treated with control diet and atorvastatin

Diet	LDL Binding Constants	
	B_{max}	K_d
	μg/mg	
Control (n = 11)	2.28 ± 0.63[b]	13 ± 4
Atorvastatin (n = 16)	2.95 ± 0.78[a]	9 ± 2

Data are presented as mean ± SD. Samples from atorvastatin groups were pooled and analyzed with Student's t test. Values in the same column with different superscripts are significantly different, P = 0.03 (atorvastatin group vs. control).

TABLE 6. Activity of hepatic enzymes of guinea pigs treated with 0, 1, 3, 10, and 20 mg/kg atorvastatin

Diet	Hepatic Enzymes		
	HMG-CoA Reductase	ACAT	7α-Hydroxylase
	pmol/min-mg		
Control	3.1 ± 7.7	31 ± 16[a]	0.6 ± 0.3
AT, 1 mg/kg	1.7 ± 1.9	12 ± 2[b]	ND
AT, 3 mg/kg	1.2 ± 0.5	11 ± 8[b]	1.2 ± 0.9
AT, 10 mg/kg	1.6 ± 1.0	12 ± 3[b]	ND
AT, 20 mg/kg	2.3 ± 1.4	6 ± 5[b]	1.1 ± 0.6

Data are presented as mean ± SD; n = 6 per dietary group; n = 11 for control group. Values in the same column with different superscripts are significantly different, P = 0.0002 (one-way ANOVA); ND, not determined.

Effects of atorvastatin on hepatic enzymes

Microsomes isolated from guinea pigs treated with atorvastatin were used to measure the activity of different enzymes related to cholesterol metabolism. HMG-CoA reductase activity was not affected by atorvastatin (**Table 6**). The lack of effect of atorvastatin on the enzyme activity may be due to the inhibitor remaining associated with HMG-CoA reductase even after one wash of microsomal pellet. In addition, guinea pigs were killed in the non-fasted state to obtain measurable levels of the enzyme in control animals, therefore the drug was consumed prior to killing with the possibility of not allowing sufficient time for up-regulation of HMG-CoA reductase.

In contrast, hepatic ACAT activity was significantly decreased with drug intake. ACAT activity was reduced approximately by 61% (31 ± 16 vs. 12 ± 2) with 1, 3, and 10 mg/kg per day and by 81% (31 ± 16 vs. 6 ± 5) with 20 mg/kg per day of atorvastatin (Table 6). A reduction in ACAT activity may be due to a decrease in substrate concentration (free cholesterol) which may be related to less cholesteryl ester available for packing into VLDL. Hepatic cholesterol 7α-hydroxylase activity was not affected by atorvastatin (Table 6).

Effects of atorvastatin in VLDL-TAG and apoB secretion rates

Two doses of atorvastatin were used in order to evaluate the effects of the drug on VLDL-TAG and apoB secretion rates (3 and 20 mg/kg per day). There was a strong correlation between plasma triacylglycerol concentration and time during treatment with Triton WR-1339 injection in the 8 h of the experiment (r = 0.9) which indicated that VLDL catabolism was totally blocked. Treatment with atorvastatin had no effect on VLDL triacylglycerol secretion rates (**Table 7**). In con-

TABLE 7. Triacylglycerol and apolipoprotein B secretion rates of guinea pigs treated with 0, 3, and 20 mg/kg of atorvastatin

Diet	Secretion Rates	
	Triacylglycerols	Apolipoprotein B
	mg/kg-h	
Control	55 ± 9	1.38 ± 0.53[a]
AT, 3 mg/kg	45 ± 17	0.65 ± 0.39[b]
AT, 20 mg/kg	59 ± 9	0.33 ± 0.12[b]

Data are presented as mean ± SD; n = 6 per dietary group; n = 5 for control group. Values in the same column with different superscripts are significantly different, $P = 0.001$ (one-way ANOVA).

trast, apoB secretion rates were reduced by drug treatment ($P < 0.001$) by 59% (3 mg/kg per day) and 76% (20 mg/kg per day) compared to control animals.

Nascent VLDL number of molecules was significantly affected by atorvastatin treatment. Triacylglycerol and phospholipid number of molecules were increased by both doses compared to control group (**Fig. 3**). Triacylglycerols increased by 2- and 4-fold for 3 and 20 mg/kg per day, respectively, while phospholipids increased 2- and 3-fold for the same doses. An increase in the number of molecules after drug treatment indicates production of larger VLDL particles but smaller in number as shown by the decreased production of apoB.

DISCUSSION

HMG-CoA reductase inhibitors are a group of compounds that have been used to decrease plasma cholesterol in several trials over the last decade (2–13). There are different types of reductase inhibitors which differ among themselves on physicochemical properties including hydrophilicity, tissue specificity, and efficacy

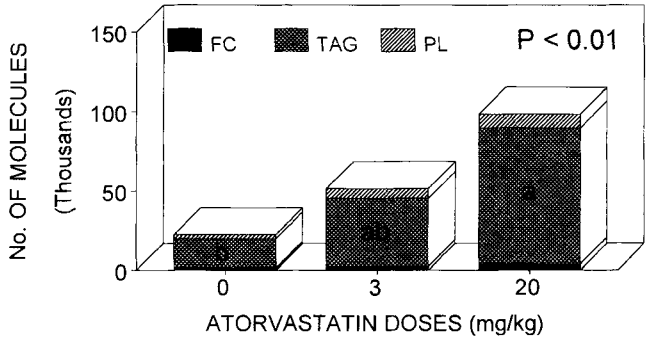

Fig. 3. Nascent VLDL number of molecules from guinea pigs fed diets containing 0, 3, and 20 mg/kg of atorvastatin. FC, free cholesterol; TAG, triacylglycerol; PL, phospholipids. The numbers of triacylglycerol and phospholipid molecules were significantly higher for the animals treated with atorvastatin ($P < 0.001$). No significant difference was found for free cholesterol number of molecules.

(34). Atorvastatin, a new HMG-CoA reductase inhibitor, has been tested in humans and several animal models (10, 11). This drug appears to be well tolerated and non-toxic as shown by different studies (9, 35). The main mechanisms of action of atorvastatin are in the process of being elucidated (11, 12). The main objective of the present study was to address the hypocholesterolemic mechanisms of atorvastatin in the guinea pig, an animal model that has been shown to respond to dietary and drug treatments by altering plasma cholesterol levels mainly in the LDL fraction (24, 36).

Effects of atorvastatin on plasma lipoproteins

Atorvastatin's main effect was a dose-dependent reduction in total plasma cholesterol levels which was accounted for by a reduction in LDL cholesterol as neither VLDL, HDL, nor plasma triacylglycerol levels were affected by drug therapy. In addition, apolipoprotein B levels in plasma was decreased in a dose-dependent manner by atorvastatin. This suggests that atorvastatin had an effect on apoB-rich lipoprotein secretion by the liver and/or lipoprotein hepatic uptake. These results agree with decreases in plasma cholesterol and apoB found in other studies including humans and animal models (7, 9, 11). In contrast to our findings, Nawrocki et al. (9) found a decrease in plasma triacylglycerol concentrations in patients treated with different doses of atorvastatin, and atorvastatin treatment also resulted in reduction in plasma triacylglycerol levels in guinea pigs fed normal chow (37).

Atorvastatin had an effect on lipoprotein composition. VLDL and LDL triacylglycerols were increased as a result of drug treatment, while cholesteryl esters were decreased in these particles. These data are similar to results found by Berglund and coworkers (36) where guinea pigs treated with lovastatin showed a decrease in the free cholesterol/triacylglycerol ratio of lipoproteins. These compositional modifications may be due to changes during hepatic production of lipoproteins or in the intravascular processing of these particles. One possible explanation for these changes may involve a reduced activity of cholesteryl ester transfer protein (CETP). Studies conducted by Ahnadi, Berthezene, and Ponsin (38) showed a reduction in CETP activity after treatment with simvastatin. Although atorvastatin's effect on CETP activity was not measured, a similar mechanism to that shown with simvastatin may have taken place. Another possibility may involve hepatic production of VLDL with less cholesteryl ester due to down-regulation of hepatic ACAT activity as discussed below.

Changes in lipoprotein composition may have an effect on the affinity of the apoB/E receptor for these particles as suggested by studies conducted by Berglund et al. (36) and Witztum et al. (39). In those studies LDL

particles isolated from guinea pigs treated with lovastatin or cholestyramine were cleared more slowly compared to LDL from control animals. In vivo studies using radiolabeled LDL isolated from control and atorvastatin-treated guinea pigs should be conducted in order to determine whether drug modifications of LDL composition affect its interaction with the apoB/E receptor.

Effects of atorvastatin on hepatic cholesterol homeostasis

Hepatic cholesteryl ester was decreased by 20 mg/kg per day of atorvastatin. It is possible that decreases in hepatic cholesteryl ester reduced the amount of cholesterol available for VLDL formation (40). The main enzymes involved in hepatic cholesterol metabolism were studied in order to determine specific effects of atorvastatin on cholesterol homeostasis. HMG-CoA reductase activity was not affected by any of the doses tested. The results found for this enzyme were unexpected as HMG-CoA reductase has been shown to be up-regulated after treatment with different HMG-CoA reductase inhibitors in the rat (41). This increase in HMG-CoA reductase represents a compensatory response to the inhibition of the enzyme by drug treatment (42). A possible explanation for the results found in this study is that during microsome isolation the inhibitor was not released from the enzyme active site. Another explanation could be that there is no up-regulation of enzyme activity in guinea pigs treated with atorvastatin. Further studies of isolation of microsomes after guinea pigs have been exposed to the drug at different time points before killing need to be conducted to clarify these issues.

Cholesterol 7α-hydroxylase activity was also determined and no effect of atorvastatin was found, similar to studies reported in rats treated with HMG-CoA reductase inhibitors (42).

Hepatic ACAT activity was significantly decreased with atorvastatin treatment. A strong correlation between ACAT activity and microsomal free cholesterol was found, confirming that ACAT activity is regulated by cholesterol in the media (43). Some studies suggest that ACAT esterifying action in the liver is important for regulation of VLDL secretion. Lower ACAT activity may lead to less cholesteryl ester available for VLDL packing, causing a reduction in VLDL secretion from the liver, as suggested by studies conducted by Carr, Parks, and Rudel in African green monkeys (44). This theory is in agreement with a reduction of cholesteryl ester in VLDL particles from drug-treated animals, similar to what was found by Shand and West (45) in rats treated with simvastatin. In addition, a decreased secretion of VLDL results in less LDL production in the plasma compartment and consequently lower plasma LDL cholesterol levels.

Hepatic apoB/E receptor number was determined in order to address the possible contribution of this pathway to the reduction in LDL cholesterol. Guinea pigs treated with atorvastatin showed an increase in the number of apoB/E receptors. Therefore, up-regulation of hepatic apoB/E receptors by atorvastatin contributed to the cholesterol lowering effect of this drug. These results are similar to those found by Berglund and coworkers (36). However, our results differ from those obtained by Auerbach et al. (11) and Burnett et al. (12) where endogenous hypercholesterolemic rabbits and miniature pigs treated with atorvastatin showed no increase in the number of receptors. Differences among these studies can be attributed to the use of different animal models and different doses of atorvastatin.

Effects of atorvastatin on VLDL secretion rates

Apolipoprotein B secretion rates are indicative of the amount of VLDL that is secreted by the liver as there is only one apoB per VLDL particle (46). Drug therapy decreased VLDL apoB secretion rates in guinea pigs, reducing the amount of VLDL secreted by the liver.

The number of molecules in nascent VLDL were determined so see whether there was a change in composition due to drug treatment. Nascent VLDL particles isolated from Triton-injected guinea pigs showed an increase in number of triacylglycerol and phospholipid molecules. This indicates a change in particle size, i.e., atorvastatin treatment produces larger VLDL particles rich in triacylglycerol. It is known that larger VLDL particles are more effectively removed from plasma than smaller VLDL particles (47), reducing the formation of LDL through the delipidation cascade.

Similar to studies in humans and different animal models treated with HMG-CoA reductase inhibitors, atorvastatin decreased cholesterol levels in the guinea pig mainly by reducing the LDL fraction in plasma. The present study conducted in guinea pigs suggests that atorvastatin's mode of action is a combination of two important pathways of cholesterol metabolism: *1*) decreased production rates of large VLDL particles with less cholesteryl ester and more triacylglycerols; and *2*) inhibition of HMG-CoA reductase activity that caused an up-regulation of the apoB/E receptor increasing LDL removal from plasma as a compensatory mechanism.

From these studies we conclude that the reduction in plasma LDL cholesterol concentrations by atorvastatin is a result of the effects of this drug on hepatic cholesterol homeostasis. The depletion of hepatic microsomal cholesterol, due to HMG-CoA reductase inhibition, reduced hepatic ACAT activity, a situation possibly related to the decreased secretion of VLDL particles containing less cholesteryl ester (44). These cholesteryl ester-

depleted VLDL are known to have decreased conversion to LDL in plasma (47). Finally, the decreases in hepatic microsomal cholesterol up-regulated apoB/E receptor activity and increased LDL catabolism. Future studies will address whether compositional differences in lipoproteins induced by atorvastatin contribute to the hypocholesterolemic action of this HMG-CoA reductase inhibitor.

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