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Hypocholesterolemic Effects of 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitors in the Guinea Pig

ATORVASTATIN VERSUS SIMVASTATIN

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ABSTRACT. Male Hartley guinea pigs were fed a hypercholesterolemic diet rich in lauric and myristic acids with 0, 10, or 20 mg/kg of simvastatin or atorvastatin for 21 days. Atorvastatin and simvastatin resulted in a lowering of plasma low-density lipoprotein (LDL) cholesterol in a dose-dependent manner by an average of 48 and 61% with 10 and 20 mg/kg, respectively. Both statins were equally effective in lowering plasma LDL cholesterol and apolipoprotein B (apo-B) levels. Atorvastatin and simvastatin treatments yielded LDL particles that differed in composition from the control. Due to the relevance of LDL oxidation and cholesteryl ester transfer in plasma to the progression of atherosclerosis, these parameters were analyzed after statin treatment. Atorvastatin and simvastatin treatment decreased the susceptibility of LDL particles to oxidation by 95% as determined by the formation of thiobarbituric acid reactive substances. An 80% decrease in the transfer of cholesteryl ester between high-density lipoprotein (HDL) and the apo-B-containing lipoproteins was observed after simvastatin and atorvastatin treatment. In addition, statin effects on plasma LDL transport were studied. Simvastatin- and atorvastatin-treated guinea pigs exhibited 125 and 175% faster LDL fractional catabolic rates, respectively, compared with control animals. No change in LDL apo-B flux was induced by either treatment; however, LDL apo-B pool size was reduced after statin treatment. Hepatic microsomal free cholesterol was lower in the atorvastatin and simvastatin groups. However, only atorvastatin treatment resulted in an 80% decrease of acyl-CoA:cholesterol acyltransferase activity ($P < 0.001$). In summary, atorvastatin and simvastatin had similar LDL cholesterol lowering properties, but these drugs modified LDL transport and hepatic cholesterol metabolism differently. BIOCHEM PHARMACOL **58**;7:1209–1219, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. atorvastatin; simvastatin; LDL oxidation; LDL fractional catabolic rates (FCR); guinea pig; CETP; ACAT

Endogenous cholesterol synthesis accounts for approximately 65% of total cholesterol in the body, the extrahepatic tissues being the major contributors to this production [1]. Inhibition of HMG-CoA§ reductase, the main regulatory enzyme of cholesterol synthesis, results in an up-regulation of the LDL receptor as a compensatory mechanism. The overall effect of decreasing reductase activity is a reduction in total plasma and LDL cholesterol levels [2]. Statins, HMG-CoA reductase inhibitors, have been developed as a tool to decrease plasma cholesterol levels in hypercholesterolemic patients [3, 4].

The pharmacologic effect of statins has been proven both in animal models and in humans [5–7]. Although the molecular and kinetic mechanism of action of statins is still under investigation, these inhibitors apparently do not have an inherent difference in affinity for the enzyme. However, according to their physicochemical and pharmacokinetic characteristics, the effects are somewhat different between compounds [8]. Atorvastatin, one of the newest HMG-CoA reductase inhibitors, is highly liver-selective and has a long duration of action [9] in both humans and rats [10].

Since the development of the first reductase inhibitor, efforts have been made to elucidate the precise mechanisms by which statins reduce LDL cholesterol. However, there is still controversy regarding the effects of statins on LDL clearance and apo-B production by the liver. Studies in humans and animals have had contradictory results regarding this issue. While some studies indicated that statins reduce LDL cholesterol only by decreasing the secretion of

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§ *Abbreviations*: ACAT, acyl-CoA:cholesterol acyltransferase; apo-B, apolipoprotein B; AT-LDL, atorvastatin low-density lipoprotein; C-LDL, control low-density lipoprotein; CETP, cholesterol ester transfer protein; FCR, fractional catabolic rate; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; MDA, malonaldehyde; Sim-LDL, simvastatin low-density lipoprotein; TAG, triacylglycerol; TBARs, thiobarbituric reactive acid substances; and VLDL, very-low-density lipoprotein.

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apo-B-containing lipoproteins from the liver [11–13], others demonstrated a role for the LDL receptor in increasing LDL clearance [14–16]. In a previous study in guinea pigs [17], we found that atorvastatin causes both a decrease in VLDL–apo-B secretion rates and an increase in the number of hepatic apo-B/E receptors.

The present study was designed to further investigate the effects of high doses of atorvastatin and simvastatin on some aspects of cholesterol and lipoprotein metabolism. A comparison between statins was performed to better define the mechanisms responsible for plasma cholesterol reduction and to determine differences in efficiency. Of particular interest was the effect of statins on LDL transport and hepatic cholesterol metabolism. In addition, the effects of these compounds on LDL oxidation and CETP activity were tested due to the relationship between these two variables and atherosclerosis [18, 19].

For these studies, the guinea pig was used as the animal model because of similarities to humans regarding cholesterol and lipoprotein metabolism. Important similarities include LDL as the major cholesterol carrier, and response to statin treatment by decreasing plasma LDL cholesterol concentrations [17, 20].

MATERIALS AND METHODS

Materials

Cholesterol oxidase, cholesterol esterase, peroxidase, and cholesterol kits were purchased from Boehringer Mannheim. Phospholipid and free cholesterol kits were obtained from Wako. The TAG kit was obtained from the Sigma Chemical Co., and ^{125}I from Amersham. ^{131}I and Liquiflor were purchased from New England Nuclear Research Products, and cellulose propionate tubes from Beckman. Halothane was obtained from Halocarbon, and oleoyl-[1-^{14}C]coenzyme A (1.8 GBq/nmol) from Amersham. Malonaldehyde bis(diethyl acetal) was purchased from the Aldrich Chemical Co. Simvastatin and atorvastatin were provided by the Parke-Davis Research Division of the Warner-Lambert Co.

Diets

Diets were prepared and pelleted by Research Diets, Inc. Isocaloric diets were designed to cover guinea pig nutritional requirements. All diets had equal compositions except for the amount of drug. The simvastatin concentrations in the different diets were 0.0, 0.05, and 0.1%, corresponding to 0, 10, and 20 mg/kg per day. In a second study, the drugs used were simvastatin and atorvastatin at a concentration of 0.1% or 20 mg/kg per day. The amount of cholesterol in the diet was adjusted to 0.04%, an amount equivalent to 112 mg/1000 kcal or less than 300 mg/day for a human diet [21]. The fat mix was olive oil:palm kernel oil:safflower oil (1:2:1.8), diets rich in lauric and myristic acids being known to cause endogenous hypercholesterolemia in guinea pigs [17]. 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 Harlan Sprague-Dawley Inc. Animals were assigned randomly to one of the different diets for 3 weeks. This period of time has been found to be enough to reach a steady state in plasma cholesterol levels. Three animals were placed in each metal cage, and they were kept in a light cycle room (light from 7:00 a.m. to 7:00 p.m.). Animals had free access to the diet and water. Diet was removed approximately 30 min before blood collection unless otherwise indicated. 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 Institutional Animal Care and Use Committee.

Lipoprotein Isolation

Blood was obtained by heart puncture from guinea pigs anesthetized under halothane vapors. Plasma samples were collected, and a preservation mixture was added to the samples (aprotinin, 0.5 mL/100 mL; PMSF, 0.1 mL/100 mL; and sodium azide, 0.1 mL/100 mL). Plasma samples were used for lipid concentration analysis and lipoprotein isolation.

Lipoprotein isolation was done by sequential ultracentrifugation [22] in an L8-M ultracentrifuge (Beckman Instruments). VLDL was isolated at a density of 1.006 g/mL at 125,000 *g* at 15° for 19 hr in a Ti-50 rotor. LDL was isolated in a density range of 1.019 to 1.09 g/mL in quick-seal tubes at 15° for 18 hr at 125,000 *g* [23]. LDL samples were dialyzed in 0.09% NaCl, 0.01% EDTA, pH 7.2, for 24 hr and stored at 4° for composition analysis.

Plasma and Hepatic Lipids

Plasma samples were analyzed for cholesterol and TAG using enzymatic methods [24] and for HDL cholesterol. HDL cholesterol was analyzed after precipitation of apo-B-containing lipoproteins with dextran sulfate [25].

Livers, excised from guinea pigs after exsanguination, were stored at −20° for lipid analysis. Lipid extraction was performed according to Carr *et al.* [26]. Briefly, 1 g of liver was sliced into small pieces and combined with 10 mL of chloroform:methanol (2:1) overnight. Lipid extraction was accomplished 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 homogenized in 0.2 mL of ethanol for enzymatic determination of total and unesterified cholesterol.

Hepatic Microsomal Lipids

Free cholesterol was assayed in hepatic microsomes isolated from guinea pigs fed with the different diets. For lipid extraction, microsomes (2–3 mg protein) were treated with 20 vol. of chloroform:methanol (2:1) according to Folch *et al.* [27]. Samples were dried under nitrogen, and lipids were solubilized with 1 mL of Triton X-100 (1%). Free cholesterol was determined by enzymatic methods.

Plasma CETP

CETP activity (transfer rate of cholesteryl ester from HDL to apo-B-containing lipoproteins) was measured in plasma samples from control and simvastatin-treated guinea pigs under physiological conditions as reported by Fernandez *et al.* [28]. Briefly, plasma samples (duplicates) were incubated at 37°, and the changes in HDL cholesteryl ester mass were determined over a period of 6 hr in fresh plasma samples. LCAT activity was inhibited by the addition of DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) [29]. HDL, total, and free cholesterol were analyzed by enzymatic methods. CETP activity was calculated as the decrease in cholesteryl ester in HDL in samples incubated for 6 versus 0 hr or control. Results are expressed as micrograms per milliliter per hour.

Determination of apo-B Concentration

Polyclonal antibodies to apo-B were obtained by injection of guinea pig purified LDL into a sheep. Antibodies were purified by affinity column. The concentration of apo-B was determined for plasma samples using the radioimmunodiffusion assay [30], in which the antigen is 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 into 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 a standard. Plates were incubated at 37° for 72 hr. 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 apo-B concentrations in samples [30].

Lipoprotein Characterization

VLDL and LDL were analyzed for phospholipids, TAGs, free cholesterol, and total cholesterol by enzymatic methods [24]. Cholesteryl ester was calculated as the difference between total cholesterol and free cholesterol times 1.67, which represents the fatty acid fraction. Protein was measured by the Markwell modification of the method of Lowry [31].

In Vitro *LDL Susceptibility to Oxidation*

LDL from individual samples was isolated using agarose–heparin affinity columns (LDL-direct method) and dialyzed in EDTA-free PBS (10 mM $NaPO_4$ buffer, pH 7.4, containing 0.15 M NaCl). Aliquots of 500 μL were incubated in the presence of $CuCl_2$ to induce oxidation. Incubation was done at either 37° or 4° for 3 hr. The lipid peroxide content for oxidized LDL was determined by analyzing TBARs expressed as MDA equivalents [32]. The TBARs reaction was conducted by addition of 2 mL of TBARs reagent (26 mM TBA and 0.92 M trichloroacetic acid in 0.25 N HCl) to the samples after their respective incubations. After TBARs reagent addition, samples were incubated for 15 min in boiling water, and 2.5 mL of *n*-butanol was added. Phases were separated by centrifugation at 2600 *g* for 20 min. The pink color developed in the organic layer was read in a spectrophotometer at 532 nm. A standard curve from 0 to 16 nmol of MDA was prepared by serial dilution of a stock solution with an initial concentration of 16 μM MDA.

Hepatic Microsome Isolation

Microsomes were isolated as previously described [33]. Briefly, livers were pressed through a tissue grinder, placed in cold buffer (50 mmol/L of KH_2PO_4, 0.1 mol/L of sucrose, 50 mmol/L of KCl, 50 mmol/L of NaCl, 30 mmol/L of EDTA, and 2 μmol/L of 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), followed by 1 hr centrifugation at 100,000 *g* at 4°. Samples were centrifuged further for an additional hour at 100,000 *g*, homogenized, and stored at −70° for enzyme analysis. Protein content in microsomes was measured according to Markwell *et al.* [31].

ACAT Activity

Hepatic ACAT (EC 2.3.1.26) activity was measured according to Smith *et al.* [34]. No exogenous cholesterol was added. Hepatic microsomes (0.8 to 1.0 mg protein per assay) were preincubated with albumin (84 mg/mL) and buffer (50 mmol/L of KH_2PO_4, 1 mol/L of sucrose, 50 mmol/L of KCl, 30 mmol/L of EDTA, and 50 mmol/L of NaF) to a final volume of 0.18 mL for 5 min at 37°. Oleoyl[1-^{14}C]coenzyme A (500 μmol/L; 0.15 Gbq/pmol) was added, and the samples were incubated for 15 min at 37°. 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. An 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

TABLE 1. Plasma lipids and CETP activity of guinea pigs treated with 0, 10, and 20 mg/kg of atorvastatin or simvastatin

Treatment (mg/kg)	Plasma lipids (mg/dL)			Plasma CETP activity (μg/m1 · hr)
	TPC	TAG	apo-B	
Control (0)	92 ± 21[a]	152 ± 92	63 ± 24[a]	15 ± 13[a]
AT (10)	49 ± 12[b]	97 ± 22	35 ± 8[b]	ND*
Sim (10)	47 ± 14[b]	128 ± 44	22 ± 12[b]	3 ±2[b]
AT (20)	35 ± 10[b]	101 ± 26	21 ± 12[b]	4 ± 2[b]
Sim (20)	37 ± 12[b]	174 ± 89	22 ± 4[b]	2 ± 1[b]

Data are presented as means ± SD; N = 17 for the control group (0), N = 6 per dietary group with drug treatment. Data in the control group are an average from controls in the present study and controls from a previous publication [17]. Values in the same column with different superscripts, are significantly different, $P < 0.05$ (one-way ANOVA). Abbreviations: TPC, total plasma cholesterol; TAG, triacylglycerol; apo-B, apolipoprotein B; AT, atorvastatin; Sim, simvastatin; and CETP, cholesteryl ester transfer protein.
* ND, not determined.

containing 30 μg of 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 and scraped from the plate, and radioactivity was counted. Recoveries of [^{3}H]cholesteryl oleate were between 70 and 90%.

In Vivo *LDL Kinetics*

LDL kinetics were studied to accomplish two objectives: (a) to determine the effect of HMG-CoA reductase inhibitors on LDL uptake, and (b) to determine whether compositional changes in LDL associated with statin treatment would affect LDL FCR. LDL isolated from control or drug-treated guinea pigs was radioiodinated by the iodine monochloride method of Goldstein *et al.* [35]. Two different isotopes were used to label the different LDL particles. ^{125}I was used to radiolabel control LDL (C-LDL), which is LDL isolated from control animals, and ^{131}I was used for drug LDL, which is LDL isolated from animals treated with simvastatin (Sim-LDL) or with atorvastatin (AT-LDL). C-LDL was labeled with ^{125}I due to its longer half-life, which would facilitate the use of this radioisotope. No major difference due to the isotope used for each particle was expected, as reported by Berglund *et al.* [14]. Lipoproteins were used within 2 days to minimize possible changes due to radiation oxidation [36].

Twenty guinea pigs were used in this experiment. Eight guinea pigs were placed in the control group, six were treated with simvastatin, and six with atorvastatin. From the eight animals in the control group, four were injected with a mixture of C-LDL and Sim-LDL. The mixture of C-LDL or drug-LDL was injected through an indwelling catheter via the carotid artery at a concentration of 70 μg of LDL protein. Plasma samples were taken at 5 min and 0.5, 1, 2.5, 5, 10, 22, and 28 hr after injection. Plasma disappearance of radiolabeled control and drug LDL was followed by counting plasma samples directly in the gamma counter. LDL FCR values were determined following a two-pool model as described by Matthews [37].

The apo B concentration was measured as described above, and LDL apo-B pool size was calculated by multiplying apo-B concentration in mg/dL × plasma volume adjusted to 1 kg of animal weight. Plasma volume was assumed to be 4% of guinea pig weight as reported previously [38]. LDL apo-B flux was calculated by multiplying apo-B pool size (mg/kg) × FCR (hr^{-1}).

Statistical Analysis

Data were analyzed by one-way ANOVA. *P* values less than 0.05 were considered statistically significant. The Newman-Keuls test was used for post-hoc analysis. Data are presented as means ± SD. Statistical analysis of the kinetic model data were best fitted using a two-pool model (JANA, SCI).

RESULTS

Effects of HMG-CoA Reductase Inhibitors on Plasma Lipids and Lipoproteins

No difference in weight gain was observed in guinea pigs fed the different treatments, indicating that animals consumed comparable amounts of the diet (data not shown). Simvastatin and atorvastatin produced a similar hypocholesterolemic effect on total plasma and LDL cholesterol (Tables 1 and 2). Both statins reduced plasma total cholesterol in a dose-dependent manner, and this was due entirely to decreases in the LDL fraction. Both statins were equally effective in reducing total and LDL cholesterol levels. Plasma total cholesterol was reduced in a dose-dependent manner by atorvastatin and simvastatin (47 and

TABLE 2. Lipoprotein cholesterol distribution in guinea pigs treated with 0, 10, or 20 mg/kg of simvastatin or atorvastatin

Treatment (mg/kg)	Cholesterol (mg/dL)		
	VLDL	LDL	HDL
Control (0)	4 ± 3	78 ± 8[a]	17 ± 2
AT (10)	2 ± 1	39 ± 11[b]	9 ± 3
Sim (10)	3 ± 1	30 ± 12[b]	15 ± 4
AT (20)	1 ± 0	26 ± 8[b]	7 ± 2
Sim (20)	4 ± 2	21 ± 7[b]	11 ± 4

Data are presented as means ± SD; N = 17 for the control group (0), N = 6 per dietary group with drug treatment. Data in the control group are an average from controls in the present study and controls from a previous publication [17]. Values in the same column with different superscripts are significantly different, $P < 0.05$ (one-way ANOVA).

TABLE 3. Composition of LDL isolated from guinea pigs treated with 0, 10, and 20 mg/kg of atorvastatin or simvastatin

Treatment (mg/kg)	LDL components (%)				
	FC	CE	TAGs	PL	PRO
Control (0)	1.6 ± 1.1	56 ± 6^{a}	8 ± 2^{a}	7 ± 4^{a}	26 ± 5
AT (10)	2.2 ± 1.2	51 ± 9^{a}	9 ± 3^{ab}	9 ± 3^{a}	28 ± 1
Sim (10)	1.4 ± 0.5	59 ± 2^{a}	10 ± 2^{ab}	8 ± 1^{a}	22 ± 2
AT (20)	2.8 ± 0.4	41 ± 3^{b}	20 ± 9^{c}	15 ± 1^{b}	21 ± 8
Sim (20)	1.6 ± 0.5	54 ± 6^{a}	14 ± 6^{b}	9 ± 1^{a}	21 ± 4

Data are presented as means ± SD; N = 17 for the control group (0), N = 6 per dietary group with drug treatment. Data in the control group are an average from controls in the present study and controls from a previous publication [17]. 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, triacyglycerol; PL, phospholipids; PRO, protein; AT, atorvastatin; and Sim, simvastatin.

62% and 49 and 59%, 10 or 20 mg/kg per day, respectively). LDL cholesterol was reduced between 50 and 66% with atorvastatin and by 61 and 73% with 10 and 20 mg/kg per day of simvastatin, respectively ($P < 0.001$). VLDL and HDL cholesterol were not affected by either drug at the different doses tested (Table 2). In contrast to clinical findings, atorvastatin and simvastatin treatment did not reduce plasma TAG concentrations significantly in the guinea pig. Similar to atorvastatin treatment, simvastatin treatment resulted in a 44 and 66% decrease in the concentration of apo B after 10 and 20 mg/kg, respectively (Table 1).

The transfer rate of cholesteryl ester from HDL to apo-B-containing lipoproteins was 78 and 84% with 10 and 20 mg/kg of simvastatin compared with the transfer in control guinea pigs (Table 1, $P < 0.05$). Similar to simvastatin, 20 mg/kg of atorvastatin decreased the transfer rate compared with control animals (Table 1).

In contrast to atorvastatin, simvastatin treatment had no effect on the composition of VLDL (data not shown), whereas LDL TAGs were increased after 20 mg/kg of simvastatin (Table 3). However, atorvastatin treatment yielded smaller LDL particles depleted of cholesteryl esters and enriched in TAGs and phospholipids. LDL susceptibility to oxidation, measured by TBARs after 3 hr of incubation at 37° in the presence of Cu^{2+}, was decreased by 95% after statin treatments ($P < 0.001$) (Fig. 1). LDL oxidation values, represented as nmol MDA/mg of non-HDL protein, were 6.84 ± 0.95, 0.34 ± 0.41, and 0.17 ± 0.20 for control, atorvastatin, and simvastatin groups, respectively.

Effects of HMG-CoA Reductase Inhibitors on Hepatic Cholesterol and Enzymes

Atorvastatin treatment (20 mg/kg) resulted in a decrease in hepatic total cholesterol, whereas simvastatin treatment had no effect on this parameter (Fig. 2). Contrary to what was found previously [17], hepatic esterified cholesterol was not statistically different among treatment groups as compared with the control. These results may be due to the variability among experiments. Neither hepatic free nor esterified cholesterol was affected after statin treatments compared with the control group. However, atorvastatin groups had lower hepatic free cholesterol compared with simvastatin groups. These results suggest a stronger effect of atorvastatin on hepatic cholesterol, which may be related to ACAT activity as mentioned below. However, hepatic microsomal free cholesterol was decreased after both simvastatin and atorvastatin treatment. Both statins caused a reduction of 30 to 65% in microsomal free cholesterol compared with the control group (Fig. 3).

Microsomes isolated from guinea pigs treated with atorvastatin or simvastatin were used to measure ACAT activity. Atorvastatin treatment caused a dose–response decrease in ACAT activity, whereas simvastatin treatment had no

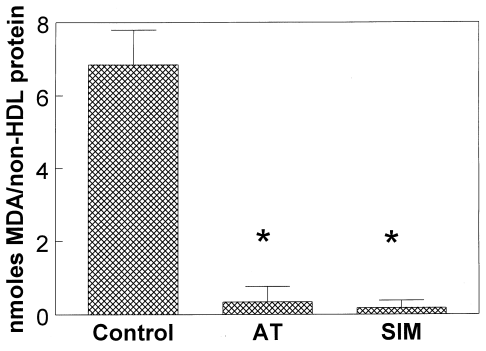

FIG. 1. *In vitro* LDL susceptibility to oxidation presented as nanomoles of MDA equivalents per milligram of non-HDL protein after 20 mg/kg of atorvastatin (AT) or simvastatin (SIM) treatment. Key: (*) $P < 0.001$ vs control.

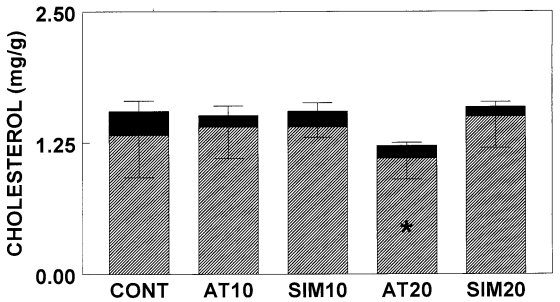

FIG. 2. Hepatic free cholesterol (solid bars) and esterified cholesterol (striped bars) concentrations in guinea pigs treated with 10 or 20 mg/kg of atorvastatin (AT) or simvastatin (SIM). Data in the control group are an average from controls in the present study and from a previous publication [17]. Key: (*) significantly different from other groups ($P < 0.001$).

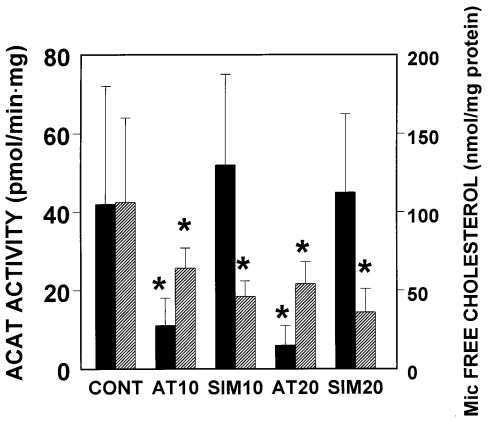

FIG. 3. ACAT activity (picomoles per minute per milligram) (solid bars) and microsomal free cholesterol concentrations (nanomoles per milligram of protein) (striped bars) from guinea pigs treated with 10 or 20 mg/kg of atorvastatin (AT) or simvastatin (SIM). Data in the control group are an average from controls in the present study and from a previous publication [17]. Key: (*) significantly different from the control ($P < 0.005$).

effect (Fig. 3). The results found in microsomal free cholesterol and ACAT activity after the use of statins suggest differences between atorvastatin and simvastatin. Both statins decreased microsomal free cholesterol, but only atorvastatin reduced hepatic ACAT activity.

Comparative results suggest that these HMG-CoA reductase inhibitors have different effects on cholesterol parameters after reduction of cholesterol synthesis. Independent of the mechanism, it appears that both drugs are equally efficacious as hypocholesterolemic agents in the guinea pig.

Effect of HMG-CoA Reductase Inhibitors on LDL FCR

LDL FCRs were studied in animals treated with simvastatin or atorvastatin. The effect of these HMG-CoA reductase inhibitors on LDL clearance was analyzed as well as the effect of particle composition on LDL kinetics. Effects on the apo-B/E receptor were studied by injecting C-LDL into guinea pigs from the control group and the simvastatin- and atorvastatin-treated groups. Particle composition effect was studied by injecting control and treated animals simultaneously with C-LDL and drug-LDL. C-LDL was cleared faster in drug-treated animals compared with control guinea pigs (Table 4, $P < 0.001$). The FCR of C-LDL particles was increased by 125 and 175% in guinea pigs treated with simvastatin or atorvastatin, respectively. When the effect of particle composition was analyzed, there was no difference between atorvastatin-treated and control animals, meaning that C-LDL and AT-LDL were cleared at the same rate in atorvastatin-treated and control animals. Contrary to these results, Sim-LDL was cleared faster than C-LDL in control animals, possibly due to LDL compositional changes that may have affected the affinity of the receptor for the particle (Table 4). However, Sim-LDL and C-LDL were cleared at the same rate in simvastatin-treated animals. When the autologous tracers were injected into control, atorvastatin-treated, or simvastatin-treated guinea pigs, both statin groups presented faster FCR compared with the control group. In addition, simvastatin- and atorvastatin-treated animals presented similar FCR (0.19 ± 0.01 vs 0.21 ± 0.03, respectively) when injected with autologous LDL particles.

A reduction of 46% in plasma cholesterol was achieved after treatment with atorvastatin and simvastatin (Table 5). Such reduction was correlated strongly with an increase in

TABLE 4. LDL fractional catabolic rates (pools/hr) of control or drug-treated guinea pigs injected with control or drug LDL particles

	LDL FCR (pools/hr)		
	Particles injected		
Treatment (N)	C-LDL	Sim-LDL	AT-LDL
Control (7)	0.08 ± 0.01^{c}	$0.11 \pm 0.01^{b,*}$	0.08 ± 0.01^{b}
Simvastatin (4)	0.17 ± 0.01^{b}	0.19 ± 0.01^{a}	
Atorvastatin (5)	0.22 ± 0.02^{a}		0.21 ± 0.03^{a}

Guinea pigs were treated with 0 or 20 mg/kg of atorvasttin or simvastatin.

Data are presented as means ± SD. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman-Keuls post-hoc test ($P < 0.05$). Abbreviations: FCR, fractional catabolic rates; C-LDL, LDL from conrol animals; Sim-LDL, LDL from simvastatin-treated animals; AT-LDL, LDL from atorvastatin-treated animals.

* Sim-LDL FCR was faster than C-LDDL or AT-LDL when injected into control animals ($P < 0.05$).

TABLE 5. Plasma cholesterol and kinetic parameters of LDL apo-B turnover in guinea pigs treated with 0 or 20 mg/kg of atorvastatin or simvastatin

		LDL-apo-B kinetic parameters		
Treatment (N)	TPC (mg/dL)	Pool size* (mg/kg)	Apo-Flux† (mg/kg·)	FCR (pools/hr)
Control (7)	79 ± 17^{a}	23.4 ± 5.0^{a}	1.92 ± 0.44	0.08 ± 0.01^{a}
Simvastatin (4)	41 ± 18^{b}	6.9 ± 3.9^{b}	1.53 ± 0.99	0.19 ± 0.01^{b}
Atorvastatin (5)	44 ± 6^{b}	10.4 ± 1.5^{b}	2.23 ± 0.58	0.21 ± 0.03^{b}

Data are presented as means ± SD. Values in the same column with different superscripts are significantly different, $P < 0.01$ (one-way ANOVA). Abbreviations: TPC, total plasma cholesterol; and FCR, fractional catabolic rates.

* Pool size was calculated as the plasma volume (4% of body weight) × plasma apo-B concentration.

† Apo-B Flux was calculated using the pool size × FCR of autologous LDL.

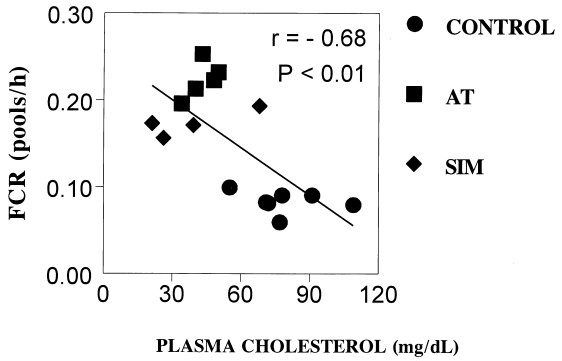

FIG. 4. Correlation between plasma total cholesterol and LDL FCR in guinea pigs injected with C-LDL. Key: (●) control group; (◆) simvastatin-treated group; and (■) atorvastatin-treated group.

LDL FCR (Fig. 4), which indicates the significant contribution of LDL clearance to the decrease observed in plasma cholesterol levels with these two statins. In addition, LDL apo-B pool size was lower after simvastatin and atorvastatin treatment (Table 5). No effect of statin treatment was observed in LDL apo-B flux compared with the control group or between drug treatments.

Overall, it appears that atorvastatin and simvastatin were equally efficacious at reducing plasma total and LDL cholesterol in the guinea pig. However, simvastatin and atorvastatin treatments resulted in different changes in hepatic cholesterol and lipoprotein composition. As shown in Fig. 5, both drugs induced similar decreases in LDL cholesterol and microsomal free cholesterol. However, atorvastatin caused compositional and size changes in LDL particles and also decreased hepatic ACAT activity. These changes may have contributed to a stronger increase in the activity of LDL receptors and a decrease in VLDL production rates (shown in our previous study [17]) induced by atorvastatin.

DISCUSSION

It is well established that the currently marketed HMG-CoA reductase inhibitors differ in physicochemical properties including lipophilicity, tissue specificity, and efficacy [39]. Atorvastatin has been tested in humans and in several animal models and has shown greater efficacy than other statins [4, 40–42]. The primary molecular and kinetic mechanisms of action of statins are still under investigation. Thus, the main objective of the present study was to address the efficacy and hypocholesterolemic mechanisms of atorvastatin in comparison with simvastatin, another widely prescribed HMG-CoA reductase inhibitor. Guinea pigs decrease plasma LDL cholesterol after HMG-CoA reductase inhibitor treatment, making them more predictive than other animal models for efficacy of statins in humans [17, 33]. In addition, guinea pigs do not inactivate reductase inhibitors rapidly, as has been observed in mice [43].

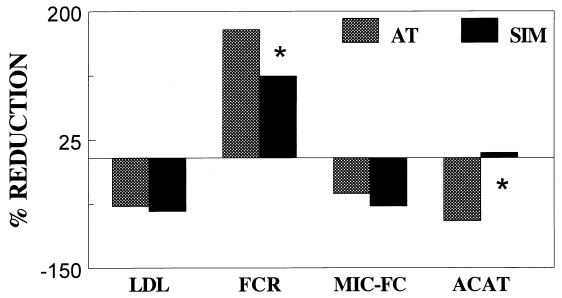

FIG. 5. Atorvastatin (AT) and simvastatin (SIM) effects on LDL cholesterol, LDL FCR, microsomal free cholesterol (MIC-FC), and ACAT activity. Data are presented as percent difference from control. Key: (*) simvastatin value is significantly different from that of atorvastatin ($P < 0.01$). Values for control guinea pigs were 78 ± 8 mg/dL for LDL cholesterol, 0.08 ± 0.01 pools/hr for LDL FCR, 47 ± 12 nmol/g for MIC-FC, and 45 ± 16 pmol/min · mg for ACAT activity.

HMG-CoA Reductase Inhibitors and Plasma Lipids and Lipoproteins

Both statins were equally efficacious in decreasing LDL cholesterol concentrations in the guinea pig. In contrast to some results obtained after simvastatin treatment in hypercholesterolemic patients [44], no effect was observed in the levels of VLDL or HDL cholesterol. However, our results are in agreement with reports on simvastatin treatment, where the concentration of apo-B decreased in parallel to LDL cholesterol [45, 46].

Intravascular processing plays an important role in the atherogenicity of lipoproteins. High doses of atorvastatin treatment caused a marked change in LDL composition in guinea pigs. LDL particles were enriched in TAG and phospholipids, whereas cholesteryl ester content was reduced. These results differ from those in patients with combined hyperlipidemia, where LDL and VLDL particles were reported to contain less TAG after atorvastatin treatment [47]. Owens and coworkers [48] reported that hypercholesterolemic patients treated with simvastatin presented reductions in the LDL esterified/free cholesterol ratio. In our studies, LDL particles isolated after simvastatin treatment had a higher percentage of TAG compared with the control, but the concentration of cholesteryl esters did not change. Modifications in LDL composition using high doses of statins in guinea pigs may relate to LDL compositional changes observed in humans after fluvastatin treatment [49].

The conversion of VLDL to LDL through the delipidation cascade as well as the rate of transfer of cholesteryl ester between HDL and the apo-B-containing lipoproteins is important in determining the concentration of VLDL and LDL in plasma. Because LDL composition was affected by drug treatment, the activity of plasma CETP was analyzed under physiological conditions to evaluate whether the rate of transfer of cholesteryl ester also was affected by these drugs. The precise role of CETP in altering lipoprotein composition is not yet clear, but it is assumed that the characteristics of LDL components found after statin treatment may be related in part to CETP activity. High doses of atorvastatin and simvastatin treatment

caused a decrease in the activity of CETP, in agreement with a study conducted by Homma and coworkers [44] in patients with hyperlipoproteinemia type II. Low CETP activity is correlated with increased levels of HDL cholesterol, the antiatherogenic lipoprotein [19]. Although there was an effect in decreasing CETP activity, a change in HDL cholesterol concentration was not detected, possibly due to the low concentrations of this lipoprotein in guinea pigs.

In addition to CETP, ACAT seems to play an important role in the amount of cholesteryl ester incorporated into the lipoproteins. The effect of statins on ACAT activity may influence lipoprotein composition as seen in our previous report with atorvastatin [17]. In addition to changes in lipoprotein composition, statin treatment had a remarkable effect in reducing LDL susceptibility to oxidation. The mechanism by which statins protect LDL from oxidation is not well known. However, Aviram *et al.* [50] reported that atorvastatin metabolites reduce LDL oxidation through free radical scavenging activity. These results provide a new insight into the effectiveness of statins for decreased formation of oxidized LDL. It is well documented that oxidized LDL plays an important role in atherosclerosis [18, 51, 52]. Decreasing the susceptibility of LDL to oxidation suggests another potential beneficial role of statins in protecting against coronary heart disease [53].

HMG-CoA Reductase Inhibitors and Hepatic Cholesterol and Enzymes

Statin treatments differed in their effects on hepatic cholesterol in the guinea pig. Atorvastatin caused a reduction in hepatic free cholesterol, whereas simvastatin had no effect. In contrast, both simvastatin and atorvastatin treatment resulted in lower free cholesterol concentrations in hepatic microsomes. In accordance with ACAT activity being regulated by the concentration of substrate in the medium [54], atorvastatin-treated animals exhibited a dose-dependent decrease in ACAT activity. Contrary to these findings, simvastatin treatment had no effect on the activity of this enzyme. Since the amount of free cholesterol in microsomes was similar after treatment with both drugs, these results suggest that the amount of ACAT protein may have been affected by atorvastatin. It is possible, but not proven, that atorvastatin treatment decreased the amount of enzyme, giving rise to a lower enzyme activity. It is very likely that the reduction in ACAT activity after atorvastatin treatment is associated with the decrease in cholesteryl esters in LDL. However, since LCAT makes a major contribution to cholesteryl esters in plasma, we cannot disregard the possibility that this enzyme might have influenced the proportion of cholesteryl esters in this lipoprotein. In addition, cholesteryl ester-depleted LDL has been correlated negatively with atherosclerosis. LDL compositional changes related to lower ACAT activity have been reported to decrease atherosclerosis in African green monkeys [55]. In agreement with this hypothesis, simvastatin treatment had no effect on ACAT activity and caused no changes in the relative concentrations of cholesteryl esters in LDL.

HMG-CoA Reductase Inhibitors and LDL FCR

It is known that statins cause an up-regulation of the LDL receptor after inhibition of HMG-CoA reductase. However, there is still disagreement regarding the contribution of LDL clearance to the cholesterol-lowering effects of these drugs. In the guinea pig, atorvastatin treatment caused an increase in the number of hepatic apo-B/E receptors [17]. In agreement with our findings, a recent report by Berglund *et al.* [15] demonstrated that hypercholesterolemic patients treated with 40 mg/day of lovastatin had a 45% higher LDL FCR compared with untreated subjects. Similar to the results of Berglund, there are other studies with hypercholesterolemic, hyperlipidemic, or normolipidemic patients that also report an increase in LDL clearance after statin treatment [19, 56, 57]. In contrast to these observations, studies in the miniature pig [58] and in hypercholesterolemic rabbits [13] indicated that atorvastatin reduced the production of apo-B-containing lipoproteins and had no effect on apo-B/E receptors. In addition, studies conducted in normolipidemic individuals and in patients with combined hyperlipidemia or familial hypercholesterolemia treated with different statins have shown a major effect in decreasing apo-B secretion [11, 12, 59].

Due to these differences in mechanism, LDL FCR was analyzed after atorvastatin and simvastatin treatment in the present study to determine the contribution of LDL clearance to plasma cholesterol lowering in the guinea pig.

When LDL isolated from control animals was injected into the three different groups, both atorvastatin and simvastatin treatment resulted in faster LDL FCR compared to the untreated group. However, atorvastatin had a more pronounced effect. These results suggest an increase in LDL receptor activity after statin treatment, in agreement with our previous report [17]. In contrast, there was no effect of atorvastatin or simvastatin on LDL apo-B flux.

To study the effect of particle composition on LDL clearance, LDL isolated from control and treated animals was injected simultaneously into guinea pigs from the three different groups. Other studies have indicated that changes in lipoprotein composition can alter the affinity of the apo-B/E receptor for such particles [14, 38]. LDL isolated from guinea pigs treated with atorvastatin was cleared at the same rate as that from control animals. However, Sim-LDL was cleared faster than C-LDL in control animals, but at the same rate in the simvastatin-treated guinea pigs. Since the LDL from the simvastatin group presented an increase in TAG proportion, this compositional change might affect the interactions of the LDL receptor with Sim-LDL. It is important to mention that when autologous LDL particles were injected into control, atorvastatin-, or simvastatin-treated guinea pigs, drug-treated animals cleared LDL faster than controls. In addition, there was no

difference in LDL FCR when drug-treated guinea pigs were injected with the homologous lipoprotein. Based on this information that represents the real life situation, one may argue about the differences observed in LDL FCR between the two drugs when C-LDL was used as a tracer.

In our previous report we found that atorvastatin led to a decreased apo-B VLDL production in guinea pigs [17]. Animals treated with atorvastatin produce fewer VLDL particles, which are larger in size, and apparently are removed faster from circulation [60]. These results suggest that the hypocholesterolemic mechanisms of atorvastatin are related to both reduced VLDL secretion and increased LDL clearance. Simvastatin treatment also resulted in increased LDL clearance. Although we did not measure VLDL production in simvastatin-treated guinea pigs, a decrease in VLDL secretion has been observed in patients with familial hypercholesterolemia treated with simvastatin [46]. In addition, studies in cells also have shown a decrease in apo-B secretion after treatment with atorvastatin or simvastatin [10].

From the results presented here, it appears that the overall effects of atorvastatin and simvastatin on cholesterol metabolism may differ. Both drugs decreased LDL cholesterol and microsomal free cholesterol to the same extent; however, the effects on hepatic free cholesterol, ACAT activity, and LDL FCR were different. Atorvastatin had a stronger effect in decreasing hepatic free cholesterol, which correlated with the lower ACAT activity, and atorvastatin treatment resulted in faster LDL FCR than simvastatin when C-LDL was used as the tracer. However, simvastatin-treated guinea pigs, when injected with the homologous LDL, had an LDL FCR similar to that of animals treated with atorvastatin. This mechanism may represent the means by which simvastatin achieves reductions in plasma LDL cholesterol similar to those of atorvastatin.

Overall, these studies suggest that atorvastatin is as potent and efficacious as simvastatin in decreasing plasma LDL cholesterol levels in the guinea pig. The distinctive pharmacologic profiles of these two statin drugs may be due primarily to differences in pharmacokinetics and in the production of active metabolites by the cytochrome P450 enzyme system, which is known to alter the primary structure of the drugs, leading to active or inactive compounds [61, 62].

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