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Acyl-Coenzyme A:Cholesterol Acyltransferase Inhibitor, Avasimibe, Stimulates Bile Acid Synthesis and Cholesterol 7α-Hydroxylase in Cultured Rat Hepatocytes and *In Vivo* in the Rat

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Acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitors are currently in clinical development as potential lipid-lowering and antiatherosclerotic agents. We investigated the effect of avasimibe (Cl- 1011), a novel ACAT inhibitor, on bile acid synthesis and cholesterol 7**α**-hydroxylase in cultured rat hepatocytes and rats fed different diets. Avasimibe dose-dependently decreased ACAT activity in rat hepatocytes in the presence and absence of **β**-migrating very low-density lipoproteins (**β**VLDL) (by 93% and 75% at 10 **μ**mol/L) and reduced intracellular storage of cholesteryl esters. Avasimibe (3 **μ**mol/L) increased bile acid synthesis (2.9-fold) after preincubation with **β**VLDL and cholesterol 7**α**-hydroxylase activity (1.7- and 2.6-fold, with or without **β**VLDL), the latter paralleled by a similar induction of its messenger RNA (mRNA). Hepatocytes treated with avasimibe showed a shift from storage and secretion of cholesteryl esters to conversion of cholesterol into bile acids. In rats fed diets containing different amounts of cholesterol and cholate, avasimibe reduced plasma cholesterol (by 52% to 71%) and triglyceride levels (by 28% to 62%). Avasimibe did not further increase cholesterol 7**α**-hydroxylase activity and mRNA in cholesterol-fed rats, but prevented down-regulation by cholate. Avasimibe did not affect sterol 27-hydroxylase and oxysterol 7**α**-hydroxylase, 2 enzymes in the alternative pathway in bile acid synthesis. No increase in the ratio of biliary excreted cholesterol to bile acids was found, indicating that ACAT inhibition does not result in a more lithogenic bile. Avasimibe increases bile acid synthesis in cultured hepatocytes by enhancing the supply of free cholesterol both as substrate and inducer of cholesterol 7**α**-hydroxylase. These effects may partially explain the potent cholesterol-lowering effects of avasimibe in the rat. (HEPATOLOGY 1999;30:491-500.)

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is the major enzyme in esterification of cholesterol, a process that plays an important role in different tissues. ACAT, located in the endoplasmic reticulum, is involved in cholesterol absorption in the intestine and in the accumulation of cholesterol in macrophages in the arterial wall. In the liver, ACAT is implicated in the storage of cholesteryl esters and the assembly and secretion of very low-density lipoproteins (VLDL).[1] Therefore, ACAT inhibitors may act as hypocholesterolemic and antiatherosclerotic agents. Recently, a novel class of ACAT inhibitors with improved bioavailability was developed, able to inhibit both intestinal and hepatic ACAT.[2] One of these ACAT inhibitors is avasimibe (CI-1011), ([[2,4,6-tris-(1-ethylethyl) phenyl] acetyl]sulfamic acid, 2,6-bis (1-methyl-ethyl)phenyl ester), which has previously been shown to have cholesterol-lowering activity and to decrease plasma triglyceride levels in animal studies.[3]

In addition to the esterification of cholesterol, which leads to its accumulation in tissues and to its secretion in lipoproteins into the circulation, an important cholesterol-metabolizing pathway in the liver is the conversion of cholesterol into bile acids. Bile acid synthesis and secretion in combination with the excretion of free cholesterol into the bile is the major route for the elimination of cholesterol from the mammalian body.[4,5] The primary route in bile acid biosynthesis in rats and humans is initiated by 7α-hydroxylation of cholesterol catalyzed by the major rate-limiting enzyme cholesterol 7α-hydroxylase, which is located in the smooth endoplasmic reticulum. This pathway predominantly causes the formation of cholate and chenodeoxycholate.[6-8] An alternative pathway in bile acid synthesis is operational as well, contributing considerably to the total bile acid synthesis in humans,[9] rats,[10] rabbits,[11] and in cultured human and rat hepatocytes.[12,13] The latter pathway is initiated by the conversion of cholesterol by the enzyme sterol 27-hydroxylase, which is located in the inner mitochondrial membrane, leading predominantly to the formation of chenodeoxycholate.[9,10,12-16] An important enzyme in this pathway is oxysterol 7α-hydroxylase.[17,18]

Inhibition of ACAT in the liver is thought to enhance the pool of free cholesterol available for elimination via the bile either directly or after conversion into bile acids. This pool of cholesterol is mostly derived from lipoproteins.[19] In addition,

Abbreviations: ACAT, Acyl-coenzyme A:cholesterol acyltransferase; VLDL, very low-density lipoprotein; LFC, low-cholesterol low-fat diet; HFC, high-fat high-cholesterol diet; mRNA, messenger RNA; WE, Williams E medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl bromide; LPDS, lipoprotein-deficient serum; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein.

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a regulatory role within bile acid synthesis has been assigned to cholesterol, which acts either as a substrate or as a factor involved in transcriptional regulation (for review see Princen et al.[20]). Differences in cholesterol supply have been shown to modulate cholesterol 7α-hydroxylase activity and gene expression in different ways in various species[21-25] including the rat.[26] In this study we investigated the effect of substrate supply after the inhibition of hepatic ACAT by avasimibe on bile acid synthesis in primary cultures of rat hepatocytes and in the rat *in vivo* under various dietary conditions.

Our data indicate that inhibition of ACAT in the liver causes a flow of free cholesterol to supply substrate for bile acid synthesis and provides a pool of regulatory cholesterol inducing cholesterol 7α-hydroxylase, without increasing the lithogenicity of the bile.

MATERIALS AND METHODS

Materials. Avasimibe (CI-1011) was kindly provided by Dr. Krause, Parke-Davis, Ann Arbor, MI. Materials used for the isolation and culturing of rat hepatocytes and assaying cholesterol 7α-hydroxylase were obtained from sources described previously.[27-29] [α-^{32}P]dCTP (3,000 Ci/mmol) and [1^{14}C]-oleate (60 mCi/mmol) were obtained from Amersham Life Sciences (Buckinghamshire, UK). 25-[^{3}H]hydroxycholesterol was obtained from NEN Life Science Products (Boston, MA). βVLDL was obtained from rabbit fed a diet containing 1% (wt/wt) cholesterol for 1 week. βVLDL was isolated from plasma by ultracentrifugation at 40,000 rpm (285,000*g*) in an SW40 rotor for 18 hours at 4°C on a NaCl density gradient composed of 4 mL plasma (density [ρ] = 1.21 with KBr) + 2.6 mL NaCl (ρ = 1.063) + 8.6 mL water.

Male Wistar rats weighing 250 to 350g were used throughout and were maintained on standard chow and water *ad libitum*. *In vitro* experiments were performed as follows: 2 days before the isolation of hepatocytes, the rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands). For the preparation of hepatocytes, the animals were killed between 9:00 AM and 10:00 AM. *In vivo* experiments were performed as follows: 4 days before starting the experiments all rats were fed a control low-cholesterol low-fat (LFC) diet, composed essentially as described by Nishina et al.[30] and purchased from Hope Farms, Woerden, The Netherlands. At the beginning of the experiment (t = 0) groups of 8 rats were transferred onto 3 different diets for 2 weeks. The 3 semi-synthetic diets were as follows: an LFC diet containing basically sucrose and nutrients; a high fat/high cholesterol diet (HFC/0) containing additionally 15% (wt/wt) cocoa butter and 1% (wt/wt) cholesterol; and a similar high fat/high cholesterol diet with additionally 0.5% (wt/wt) cholate (HFC/0.5) to facilitate intestinal uptake of fat and cholesterol and to suppress bile acid synthesis. At the beginning of the second week (t = 7 days) the diet of half of the animals in each group was mixed with 0.01% (wt/wt) avasimibe, which approximately equals a daily dose of 10 mg/kg body weight. Blood samples were taken at t = 0, 7, and 14 days. At t = 14 days animals were killed and livers were isolated to measure intracellular lipid levels, enzyme activities, and messenger RNA (mRNA) levels. Institutional guidelines for animal care were observed in all experiments.

Rat Hepatocyte Isolation and Culture. Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously.[27-29] After a 4-hour attachment period, the cell medium was refreshed with 1.0 mL (6-well plates) or 2.5 mL (dishes) of Williams E (WE) medium supplemented with 10% fetal calf serum, and cells were incubated for a further 14 hours. Avasimibe, dissolved in dimethyl sulfoxide, was added to the culture medium, between 18 and 42 hours of culture age, unless otherwise stated. The final concentration of dimethyl sulfoxide in the medium was 0.1% (vol/vol). Cells were harvested at the same time after a 42-hour culture period for measuring cellular lipid, cholesterol 7α-hydroxylase, and sterol 27-hydroxylase activity and determination of mRNA levels. Cell viability, after culturing with avasimibe, was assessed by adenosine triphosphate measurements and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl bromide (MTT) assays as described previously.[29] The latter assay depends on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO) by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically.

Quantitation of ACAT Activity and Measurement of the Mass of Intracellular and Extracellular Lipids. Cholesterol esterification was measured by determining the incorporation of [1^{14}C]-oleate (100 μmol/L, 0.25 μCi) into labeled cholesteryl oleate after the incubation of hepatocytes for 22 hours, from 18 to 40 hours of culture, with different concentrations of avasimibe in the presence or absence of βVLDL (providing 200 μg cholesterol per mL medium). After 22 hours, [1^{14}C]-oleate was added and cells were incubated for another 2 hours at 37°C. Cells were harvested at 42 hours of culture age to measure cholesterol esterification as described previously.[31]

To determine the effect of avasimibe on the mass of cellular lipids, the cells were incubated for 24 hours with or without avasimibe or βVLDL, washed 3 times with cold (4°C) phosphate-buffered saline (pH 7.4), and harvested by scraping. The cells thus collected or liver samples from the rats treated with or without avasimibe on the different diets were homogenized and samples were taken for measuring the protein content. Lipids were extracted from the cell suspension as described by Bligh and Dyer,[32] after the addition of cholesterol acetate (2 μg per sample) as an internal standard. The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 precoated plates as described.[29,31] Quantitation of the amounts was performed by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density areas using the computer program Tina version 2.09.

To determine the mass of triglycerides, free cholesterol, and cholesteryl ester secreted into the medium, the cells were incubated for 24 hours, from 26 to 50 hours of culture age, in WE medium containing 10% lipoprotein-deficient serum (LPDS) after an 8-hour preincubation period with βVLDL and extensive washing with WE medium at 37°C to remove extracellular cholesterol. At the end of the incubation period, the medium was centrifuged at 12,000 rpm (12,720*g*) for 30 minutes at 4°C to remove detached cells and cell debris. The corresponding cells were used for measuring intracellular lipid levels. Extraction and analysis of excreted lipids were the same as those described for the cell suspensions and liver samples.

Lipoprotein Analysis. Total plasma cholesterol and triglycerides were measured enzymatically using commercially available kits (Boehringer Mannheim GmbH, Mannheim, Germany). Pooled plasma of 2 rats was fractionated by FPLC analysis on a 25-mL Superose 6B column (Pharmacia AB, Uppsala, Sweden) and eluted with phosphate-buffered saline, pH 7.4.[33]

Quantitation of Mass Production of Bile Acids. Mass production of bile acids by rat hepatocytes was measured by gas-liquid-chromatography after a preincubation period of 8 hours (from 18-26 hours of culture age) with βVLDL (200 μg/cholesterol per mL medium) for the following 24-hour culture period from 26 to 50 hours in WE medium containing 10% LPDS or fetal calf serum in the presence or absence of avasimibe as described previously.[29]

Determination of Biliary Bile Acid and Cholesterol Secretion in Rats. To study the effects of avasimibe on bile formation, rats were fed an HFC/0.5 diet for 2 weeks to increase hepatic cholesterol content. After this treatment period, rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere.[34] Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. Four days after surgery, *i.e.*, after animals had regained their preoperative body weights, rats were transferred onto an HFC/0 diet (to wash-out the cholate) with or without avasimibe (0.01% wt/wt). After 1 week of treatment, the connection between both catheters was interrupted and bile was collected for 8 hours in 30-minute intervals by means of a fraction collector. Bile volume was determined gravimetrically, and samples were immediately stored at −20°C for later analysis. Bile

acids in bile were determined by an enzymatic fluorimetric assay.[35] Cholesterol in bile was measured after lipid extraction[32] as described previously.[36]

Assay of Cholesterol 7α-Hydroxylase and Sterol 27-Hydroxylase Enzyme Activity. Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cell homogenates and isolated liver microsomes were determined essentially according to Chiang,[37] measuring mass conversion of cholesterol into 7α- and 27-hydroxycholesterol. In short, 1 to 4 mg of protein of either microsomes or homogenates of cells was incubated in 1 mL of buffer containing 0.1 mol/L potassium phosphate pH 7.2, 50 mmol/L NaF, 5 mmol/L dithiothreitol (DTT), 1 mmol/L ethylenediaminetetraacetic acid, 20% glycerol (wt/vol), and 0.015 % (wt/wt) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate. Twenty microliters of 1 mg cholesterol in 45% (wt/vol) hydroxypropyl-β-cyclodextrin was added, and the mixture was incubated under agitation for 10 minutes at 37°C. Then 200 µL of a regenerating system was added containing 10 mmol/L sodium isocitrate, 10 mmol/L $MgCl_2$, 1 mmol/L nicotinamide adenine dinucleotide phosphate and 0.15 U isocitrate-dehydrogenase at 37°C. After 20 minutes of incubation, 60 µL of a stop solution containing 20% (wt/vol) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. Steroid products were oxidized at 37°C for 45 minutes after addition of 100 µL buffer containing 0.1% cholesteroloxidase (wt/vol) (Calbiochem, La Jolla, CA, #228250), 10 mmol/L potassium phosphate pH 7.4, 1 mmol/L dithiothreitol, and 20% glycerin (wt/wt), and the reaction was stopped by the addition of 2 mL ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol, and 10% chloroform (vol/vol) were analyzed by using C-18 reverse phase high-performance liquid chromatography on a Tosohaas TSK (Toso Haas, Stuttgart, Germany) gel-ODS 80TM column equilibrated with 70% acetonitril and 30% methanol at a flow rate of 0.8 mL/min. The amount of the product formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, Cambridge, UK).

Assay of Oxysterol 7α-Hydroxylase Activity. Enzyme activities of oxysterol 7α-hydroxylase in liver microsomes were determined according to Schwarz et al.[17] measuring mass conversion of 25-[^{3}H]hydroxycholesterol into [^{3}H]cholest-5-ene-3β,7α,25-triol. In short, 500 µg microsomal protein was incubated with 0.12 µmol/L 25-[^{3}H]hydroxycholesterol in a buffer containing 50 mmol/L Tris-acetate (pH 7.4), 1 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L dithiothreitol, 0.03% (vol/vol) Triton X-100, and 1.5 mmol/L nicotinamide adenine dinucleotide phosphate for 15 minutes at 37°C. The reaction was stopped by the addition of 6 mL of methylene chloride. The organic phase was evaporated under a stream of nitrogen. Residues resuspended in acetone were analyzed by thin-layer chromatography in a solvent system containing toluene/ethyl acetate (2:3).

RNA Isolation, Blotting, and Hybridization Procedures. Isolation of total RNA and subsequent electrophoresis, Northern-blotting, and hybridization techniques were performed as described previously.[28,29] The following DNA fragments were used as probes in hybridization experiments: a 1.6-kb polymerase chain reaction–synthesized fragment of rat cholesterol 7α-hydroxylase complementary DNA (cDNA), spanning the entire coding region[28]; a 1.6-kb *Hin*dIII/*Xba* I fragment of rat sterol 27-hydroxylase cDNA, a 435-bp *Pst* I fragment of hamster HMG-CoA synthase cDNA, and a 2.2-kb *Eco*RI fragment of rat LDL-receptor cDNA. As controls, a 1.2-kb *Pst* I fragment of hamster β-actin cDNA and a 1.2-kb *Pst* I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used.[29] The actin or GAPDH mRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantitated using a Phosphorimager BAS-reader (Fuji Fujix BAS 1000, Karagawa, Japan) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Statistical Analysis. Data were analyzed statistically using Student's paired (hepatocytes) or unpaired (rats) *t*-test with the level of significance selected to be $P < .05$. Values are expressed as means ± SEM.

RESULTS

Avasimibe Inhibits ACAT Activity in Cultured Rat Hepatocytes. The effect of avasimibe on ACAT activity was measured in hepatocytes in the absence or presence of an additional source of cholesterol, in the form of βVLDL, to increase the intracellular cholesterol pool. Incubation of the rat hepatocytes with avasimibe resulted in a dose-dependent decline in ACAT activity, showing a 75% inhibition at 10 µmol/L. The addition of βVLDL to the cells resulted in an increase in ACAT activity (2.5-fold) compared with control cells. ACAT inhibition by avasimibe under these conditions decreased the activity dose-dependently to the same level as in control cells (by 93%) (Fig. 1). The concentrations used in these experiments (up to 10 µmol/L of avasimibe) did not have any adverse effects on cell viability as indicated by measurements of cellular MTT and adenosine triphosphate levels (96 ± 5% and 107 ± 5%, respectively). Data are expressed as a percentage of control and are means ± SEM of independent experiments using hepatocytes from 6 rats.

To investigate the effect of ACAT inhibition by avasimibe on intracellular lipid content, the amount of free cholesterol and cholesteryl esters was measured. Incubation with βVLDL resulted in a 1.8-fold increase in cholesteryl esters in the cells (Table 1). The addition of 3 µmol/L avasimibe produced a marked decline in cholesteryl ester content in control cells (−41%) and in cells incubated with βVLDL (−55%).

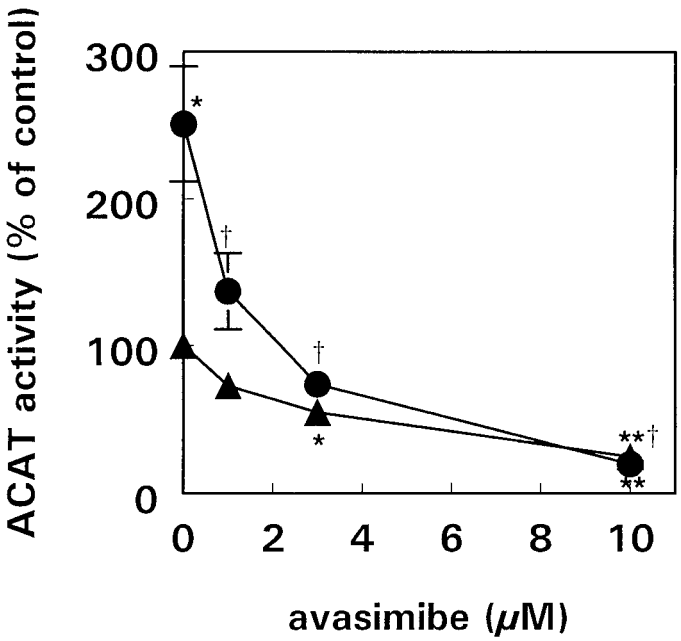

FIG. 1. Effect of avasimibe on ACAT activity. Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, in the presence or absence of avasimibe or βVLDL (200 µg cholesterol per mL medium). During the last 2 hours of the incubation [1-^{14}C]oleic acid (100 µmol/L, 0.25 µCi) was added to the cells, and at 42 hours of culture age ACAT activity was measured by determining the incorporation of [1-^{14}C]oleic acid into labeled cholesteryl oleate. Values shown are expressed as a percentage of ACAT activity in control incubations and are means ± SEM of independent experiments with hepatocytes of 3 to 5 rats. A significant difference is indicated by symbols (*$P < .05$ and **$P < .005$ compared with control cells [without incubation with avasimibe]; †$P < .001$ compared with the incubation in the presence of βVLDL without avasimibe).

TABLE 1. Effect of Avasimibe on Intracellular Lipid Levels

	FC (% of control)	CE (% of control)
Control	100	100
3 μmol/L avasimibe	95 ± 4	59 ± 8*
βVLDL	98 ± 3	183 ± 15*
βVLDL + 3 μmol/L avasimibe	108 ± 7	83 ± 5*† (45)

NOTE. Rat hepatocytes were incubated for 24 hrs, from 18-42 hrs of culture, in the presence or absence of 3 μmol/L avasimibe and an additional source of cholesterol (βVLDL, 200 μg cholesterol per mL medium). Cells were harvested and cellular cholesteryl ester (CE) and free cholesterol (FC) were measured as described in the Material and Methods. Values are expressed as a percentage of control, and are means ± SEM of independent experiments with hepatocytes from 4-6 rats. In parentheses the effect of avasimibe is given as a percentage of control with βVLDL. The mean absolute amounts, present in control cells, were 6.4 ± 0.7 μg/mg cellular protein CE and 9.4 ± 1.4 μg/mg cellular protein FC.

*$P < .05$, compared with control cells (without avasimibe).

†$P < .001$ compared with the incubation with βVLDL without avasimibe.

Avasimibe Increases Bile Acid Synthesis. To investigate the fate of the free cholesterol that becomes available after ACAT inhibition, bile acid mass production was measured. Cells were preincubated for 8 hours with βVLDL to increase the initial intracellular cholesterol content. Incubation with 3 μmol/L avasimibe resulted in a 2.9-fold increase in bile acid mass production (Fig. 2). The main bile acids formed were cholic acid and β-muricholic acid in a ratio of approximately 20:80, which did not change after incubation with the ACAT inhibitor. In the latter experiments bile acid mass production was measured after incubating the cells in WE medium supplemented with 10% LPDS. However, a similar effect of avasimibe on bile acid synthesis was found in medium with 10% fetal calf serum (data not shown).

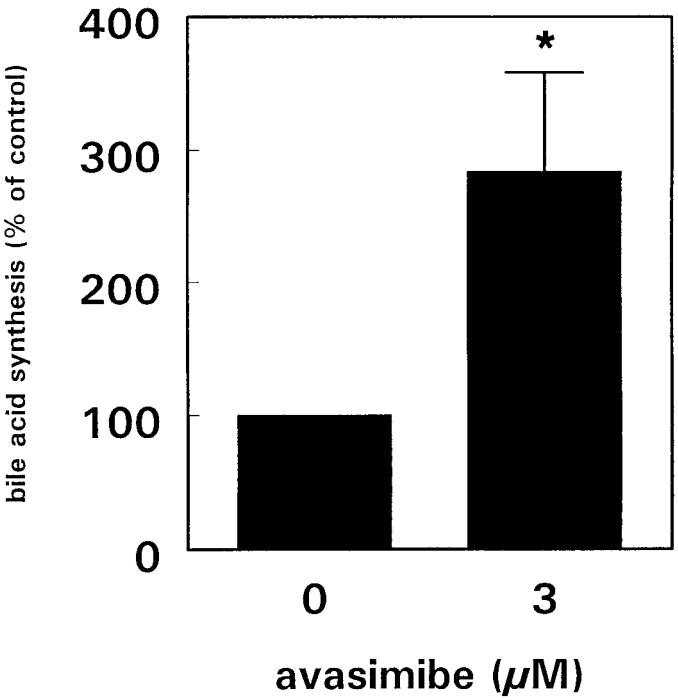

FIG. 2. Effect of avasimibe on mass production of bile acids. After an 8-hour preincubation period (from 18-26 hours of culture) with βVLDL (200 μg cholesterol per mL medium), rat hepatocytes were cultured for 24 hours in WE medium containing 10% LPDS in the presence or absence of 3 μmol/L avasimibe. Values shown are expressed as a percentage of bile acid synthesis in control incubations and are means ± SEM of independent experiments with hepatocytes from 4 rats. Absolute synthesis rate in the absence of avasimibe was 4.29 ± 0.66 μg/24 hours/mg cell protein. A significant difference between control and treated cells is indicated (*$P <$ .05).

In the cultures incubated with LPDS a cholesterol balance was determined by measuring the total amount of cellular and excreted lipids. Table 2 shows that the increase in bile acid synthesis after incubation with avasimibe is at the expense of the total amount of intracellular and secreted cholesterol mainly contained in the cholesteryl ester fraction. Intracellular triglyceride levels were significantly increased (+20 ± 6%) and excretion of triglycerides in these experiments was significantly ($P < .05$) decreased (−33 ± 6%) (data not shown). Thus, it can be shown that when cells are incubated with avasimibe, the flow of cholesterol, which is mostly derived from lipoproteins (βVLDL) shifts from mainly storage and secretion of cholesteryl esters to the conversion of cholesterol into bile acids.

Avasimibe Induces Cholesterol 7α-Hydroxylase Activity and mRNA Levels. To investigate whether changes in cholesterol 7α-hydroxylase and sterol 27-hydroxylase contribute to the increased bile acid mass production induced by avasimibe, enzyme activities and mRNA levels were determined. Rat hepatocytes were cultured in the absence or presence of βVLDL and avasimibe. βVLDL enhanced cholesterol 7α-hydroxylase activity and mRNA, 2.7- and 1.5-fold, respectively (Fig. 3). The addition of avasimibe increased cholesterol 7α-hydroxylase enzyme activity and mRNA levels in control cells 2.6- and 2-fold and further increased enzyme activity and the mRNA level in the presence of βVLDL (1.7-and 2.2-fold, with respect to the incubations containing βVLDL) (Fig. 3). The enhancing effect of avasimibe on cholesterol 7α-hydroxylase mRNA was rapid and significant after as few as 8 hours of incubation, showing a 2-fold increase (data not shown). Neither βVLDL nor avasimibe had any effect on sterol 27-hydroxylase enzyme activity (data not shown) and mRNA (Table 3).

Since the large decrease observed in ACAT activity may also have consequences for the regulatory cholesterol pool influencing the expression of LDL-receptor and cholesterol synthetic enzymes, mRNA levels of the LDL-receptor and

TABLE 2. Effect of Avasimibe on Cholesterol Balance

	Intracellular		Extracellular		
	FC (μg/mg cell protein)	CE (μg/mg cell protein)	FC (μg/mg cell protein)	CE (μg/mg cell protein)	Total Bile Acids (μg/mg cell protein)
Control	7.8 ± 2.7	6.5 ± 1.4	3.3 ± 0.5	6.1 ± 1.5	3.9 ± 0.6
3 μmol/L avasimibe	9.5 ± 3.2* (122)	3.3 ± 0.7* (51)	2.6 ± 0.5* (79)	2.4 ± 0.5* (39)	11.2 ± 2.9* (287)

NOTE. Rat hepatocytes were incubated in WE medium containing 10% LPDS for 24 hours, from 26-50 hours of culture in the presence or absence of 3 μmol/L avasimibe after a preincubation for 8 hrs with βVLDL (200 μg cholesterol per mL medium). Cells and medium were harvested to measure intra- and extracellular free (FC) and esterified (CE) cholesterol and total mass of bile acids. Values are expressed as the amount of cholesterol present as free or esterified cholesterol or as bile acids, and are means ± SEM of independent experiments with hepatocytes from 4 rats. Values between parentheses represent data expressed as a percentage of the value in control cells.

*$P < .05$.

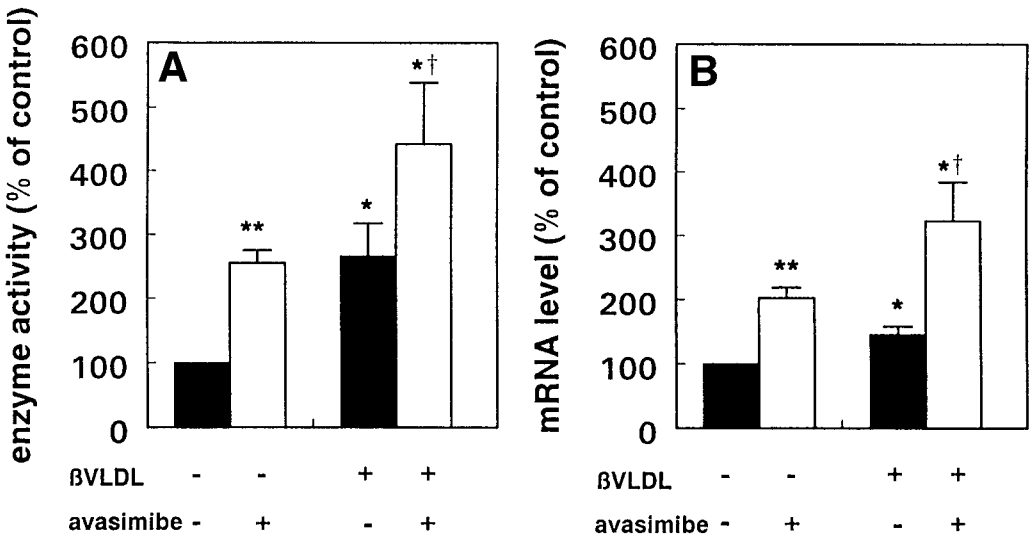

FIG. 3. Effect of avasimibe on cholesterol 7α-hydroxylase activity and mRNA levels. Rat hepatocytes were incubated for 24 hours from 18-42 hours of culture, in the presence or absence of 3 μmol/L avasimibe or βVLDL (200 μg cholesterol per mL medium). Cells were harvested after 24 hours of incubation to measure cholesterol 7α-hydroxylase activity (A) and mRNA levels (B). Values shown are expressed as a percentage of enzyme activity or mRNA levels in control cells and are means ± SEM of independent experiments with hepatocytes from 4 to 7 rats. The amount of mRNA was corrected for differences in total RNA applied to the gel, using GAPDH mRNA as an internal standard. Absolute activities of cholesterol 7α-hydroxylase in cell homogenates in the absence of avasimibe were 119 ± 18 pmol/h/mg cell protein. A significant difference between control and treated cells is indicated by symbols (*$P < .05$; **$P < .005$ compared with control cells [without incubation with avasimibe]; †$P < .05$ compared with the incubation with βVLDL without avasimibe).

HMG-CoA synthase were measured. These mRNA levels decreased on incubation with avasimibe, by 28% and 36%, respectively (Table 3). These data indicate that the inhibition of ACAT by avasimibe leads to a moderate down-regulation of genes involved in cholesterol synthesis and LDL-receptor–mediated uptake.

Avasimibe Decreases Plasma Cholesterol and Triglycerides in Rat. To investigate whether avasimibe also induces cholesterol 7α-hydroxylase *in vivo* we fed rats different semisynthetic diets with and without 0.01% (wt/wt) of avasimibe. The diets used contained no cholesterol but basically only sucrose and nutrients (LFC), a high fat/high cholesterol diet (HFC/0) containing additionally 15% (wt/wt) cocoa butter and 1% (wt/wt) cholesterol, and a similar high fat/high cholesterol diet with additionally 0.5% (wt/wt) cholate (HFC/0.5), which further increases plasma cholesterol levels by facilitating the intestinal uptake of fat and cholesterol and suppressing bile acid synthesis. Body weight and food intake did not differ according to groups (data not shown). Total plasma cholesterol levels were increased (1.5-fold) after using the HFC/0 diet as compared with the standard LFC diet and 2.4-fold with the HFC/0.5 diet (Fig. 4A). Treatment with 0.01% avasimibe completely prevented increases in total plasma cholesterol and reduced the cholesterol levels to an even significantly lower level than those found in untreated animals on an LFC diet. Triglyceride levels were clearly increased (1.9-fold) in rats on an HFC/0 diet compared with the control LFC diet. The addition of cholate to the diet (HFC/0.5) prevented this increase and reduced triglyceride levels to levels even below those of control untreated animals on the LFC diet. Animals treated with 0.01% avasimibe had reduced triglyceride levels as compared with untreated animals on the same diet (Fig. 4B). To determine the effect of avasimibe on the contribution of the various lipoproteins to the changes observed in plasma cholesterol, plasma samples were fractionated by FPLC. The increase in plasma cholesterol induced by dietary cholesterol, and the prevention of these increases by avasimibe was caused predominantly by changes in the VLDL/LDL range. Administration of avasimibe resulted in a decrease in the non-high density lipoprotein (HDL) cholesterol in all diets. HDL cholesterol changed depending on the diet fed, showing a decrease in rats fed the LFC diet and an increase in the rats fed the HFC/0.5 (data not shown).

TABLE 3. Effect of Avasimibe on Different mRNA Levels

	mRNA Level (% of control)
Cholesterol 7α-hydroxylase	202 ± 17*
Sterol 27-hydroxylase	103 ± 9
LDL-receptor	72 ± 7†
HMG-CoA synthase	64 ± 7†

NOTE. Rat hepatocytes were incubated in the presence or absence of 3 μmol/L avasimibe for 24 hours, from 18-42 hours of culture time. mRNA levels were assessed by Northern blot hybridization and scanning of the resulting phosphor-imager plates, using GAPDH as the internal standard to correct for differences in the amount of RNA applied as described in the Material and Methods. Data are expressed as a percentage of control and are means ± SEM of independent experiments using hepatocytes from 4 to 7 rats.

*$P < .01$.

†$P < .05$.

Avasimibe Decreases Hepatic Cholesteryl Ester Levels. To determine the effects of avasimibe on hepatic lipid metabolism levels of free and esterified cholesterol and triglycerides levels were measured. Feeding rats with the cholesterol-rich diet (HFC/0) did not alter the liver free cholesterol levels, but strongly increased the liver cholesteryl ester content (Table 4). The increase in cholesteryl esters was further enhanced in rats on the HFC/0.5 diet, which was now accompanied by a significant rise in liver free cholesterol. Treatment with avasimibe did not affect the cholesteryl ester content with the LFC diet, but the accumulation of cholesteryl esters was

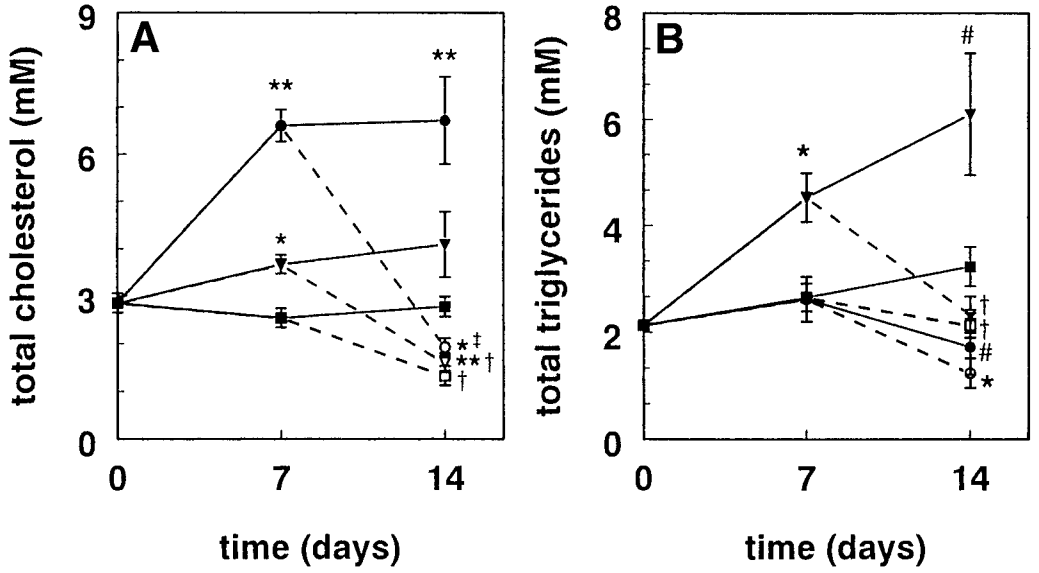

FIG. 4. Effect of avasimibe on plasma cholesterol and triglyceride levels in rats fed different diets. Animals were fed a semisynthetic diet (LFC, *squares*) or diets supplemented with 1% cholesterol/15% cacao butter (HFC/0, *triangles*) or with 1% cholesterol/15% cacao butter/0.5% cholate (HFC/0.5, *circles*) for 2 weeks (*drawn line, closed symbols*). Indicated groups received diets containing 0.01% avasimibe during the second week (*broken line, open symbols*). Blood samples were taken on day 0, 7, and 14. Plasma cholesterol (A) and triglycerides (B) were measured as described in the Material and Methods. Data represent the mean ± SEM (n = 24 on day 0, n = 8 on day 7, and n = 4 on day 14). A significant difference is indicated by symbols (*$P < .05$, **$P < .01$, and #$P = .055$ compared with control LFC diet without avasimibe treatment at the same time point; †$P < .05$, ‡$P < .01$ compared with the same diet without avasimibe treatment at the same time point.

markedly decreased with both the HFC/0 (−65%) and the HFC/0.5 (−43%) diets. Liver triglyceride levels were increased in rats on both cholesterol-rich diets (Table 4). Treatment with avasimibe tended to lower hepatic triglyceride levels in rats on the HFC/0 diet, but did not prevent the increase in triglycerides in rats on the HFC/0.5 diet.

Avasimibe Stimulates Hepatic Cholesterol 7α-Hydroxylase Activity and mRNA Levels. Addition of cholesterol to the diet (HFC/0) increased the cholesterol 7α-hydroxylase activity and mRNA levels 2.4- and 2-fold, respectively (Fig. 5). This increase was fully abolished by the simultaneous addition of cholate to this diet (HFC/0.5). Treatment with avasimibe did not affect enzyme activity and mRNA levels in rats on the control diet (LFC) or the cholesterol-rich diet (HFC/0).

TABLE 4. Effect of Avasimibe on Hepatic Cholesterol and Triglyceride Content

Diet	FC (μg/mg protein)	CE (μg/mg protein)	TG (μg/mg protein)
LFC	8.2 ± 0.2	3.2 ± 0.3	9.5 ± 0.3
+Avasimibe	8.0 ± 0.4 (96)	3.5 ± 0.7 (110)	10.3 ± 1.3 (109)
HFC/0	9.6 ± 0.8	18.7 ± 2.9*	20.1 ± 3.6†
+Avasimibe	8.2 ± 0.4 (86)	6.6 ± 0.6*‡ (35)	12.9 ± 0.9§ (64)
HFC/0.5	12.7 ± 0.8*	41.4 ± 0.9*	25.8 ± 3.9*
+Avasimibe	11.4 ± 0.4* (90)	23.7 ± 2.7*‡ (57)	24.1 ± 2.1* (93)

NOTE. Animals were treated with the indicated diet and 0.01% (wt/wt) avasimibe. In liver homogenates free cholesterol, cholesteryl esters, and triglycerides were determined using a high performance thin layer chromatography method (see Material and Methods). Data are means ± SEM (n = 4). Values between parentheses represent data expressed as a percentage of value obtained in untreated animals on the same diet. A significant difference is indicated by symbols †$P < .05$ compared with control LFC diet without avasimibe treatment. *$P < .01$ compared with control LFC diet without avasimibe treatment. ‡$P < .01$ compared with the same diet without avasimibe treatment. §$P = .1$ compared with the same diet without avasimibe treatment.

These data indicate that the amount of regulatory cholesterol available after ACAT inhibition in rats on the cholesterol-free diet LFC is too low to establish its effects on cholesterol 7α-hydroxylase. On the cholesterol-rich diet, HFC/0, the level of cholesterol 7α-hydroxylase expression cannot be further enhanced by increasing the pool of regulatory cholesterol by avasimibe treatment. However, the addition of avasimibe to the HFC/0.5 diet showed a marked increase in cholesterol 7α-hydroxylase activity and mRNA (both 2.9-fold) indicating that ACAT inhibition under conditions of sufficient cholesterol supply can counteract the down-regulation of cholesterol 7α-hydroxylase expression caused by cholate.

Sterol 27-hydroxylase mRNA levels did not change either under the various dietary conditions or after treatment with avasimibe. The oxysterol formed by sterol 27-hydroxylase can be further converted by oxysterol 7α-hydroxylase, which is located in the endoplasmic reticulum.[17,18] We also measured the activity of this enzyme to investigate whether expansion of the regulatory pool of cholesterol by avasimibe may have other effects on the alternative pathway. We could not detect significant differences in oxysterol 7α-hydroxylase enzyme activity (Table 5) by feeding various diets or avasimibe, indicating that 2 important enzymes in the alternative pathway are not affected by the different diets and inhibition of ACAT. Treatment of rats with dietary cholesterol (HFC/0 and HFC/0.5) decreased LDL-receptor mRNA levels (by 40% to 50%), but no further down-regulation was observed after treatment with 0.01% avasimibe (data not shown).

Avasimibe Does Not Affect the Lithogenicity Index of the Bile. To investigate the effect of avasimibe on the overall process of bile acid synthesis, biliary bile acid output was measured in rats fed an HFC/0 diet with or without (0.01 % wt/wt) avasimibe for 1 week after a pretreatment period for 2 weeks with HFC/0.5 to increase the initial hepatic cholesterol

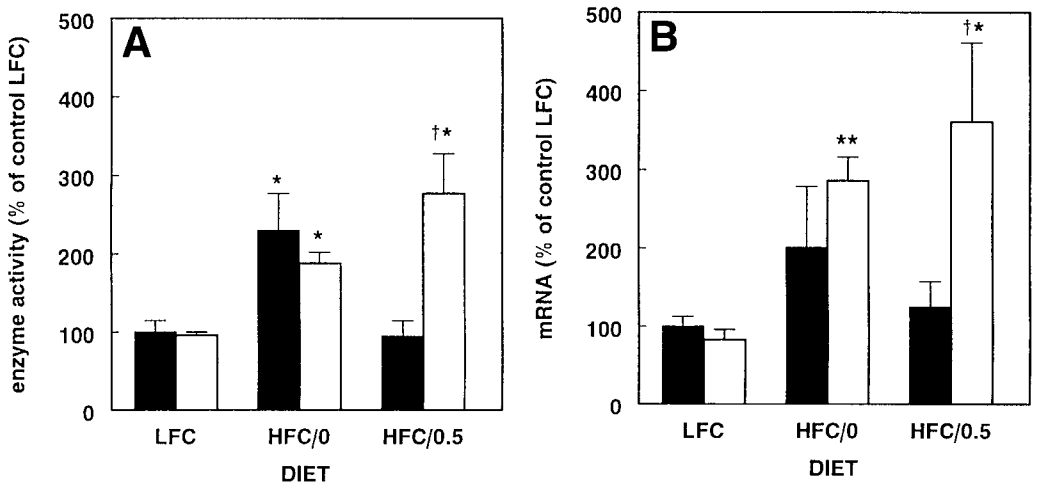

FIG. 5. Effect of avasimibe on cholesterol 7α-hydroxylase activity and mRNA levels in the rat under various dietary conditions. Cholesterol 7α-hydroxylase activity in liver microsomes (A) and mRNA levels (B) were measured in rats fed on various diets and treated with or without 0.01% (wt/wt) avasimibe. Data shown are means ± SEM (n = 4 per group). Absolute values for cholesterol 7α-hydroxylase activity were 974 ±143 pmol/h/mg protein for control LFC diet. *Closed bars*: diet only; *open bars*: diet + avasimibe. A significant difference is indicated by symbols (*$P < .05$ and **$P < .01$ compared with control LFC diet without avasimibe treatment; †$P < .05$ compared with the same diet without avasimibe treatment).

content. After exhaustion of the bile acid pool biliary output of bile acids, which reflects their *de novo* synthesis, was not further increased in the avasimibe-treated compared with control-treated group (85.9 ± 17.4 nmol/min/100 g body weight versus 77.8 ± 10.4 nmol/min/100 g body weight, respectively) (n = 4). We also determined whether inhibition of ACAT resulted in a quantitatively greater biliary cholesterol secretion compared with the amount of cholesterol converted to bile acids, which may lead to supersaturation of bile. In the initial 2 hours after interruption of the enterohepatic circulation there was no change in the molar ratio between cholesterol and bile acids excreted after avasimibe treatment compared with control rats ($4.37 \times 10^{-3} \pm 0.41 \times 10^{-3}$ vs. $4.31 \times 10^{-3} \pm 0.88 \times 10^{-3}$, respectively; n = 4). Thus, treatment with avasimibe does not lead to supersaturation of the bile.

DISCUSSION

This study shows that avasimibe increases bile acid synthesis by up-regulation of cholesterol 7α-hydroxylase expression in cultured rat hepatocytes both in control cells as well as in cells in which the intracellular cholesterol pool is increased by the addition of βVLDL. Cholesterol balance experiments showed that the inhibition of ACAT causes a change in the metabolic pathway of cholesterol by reducing lipid secretion and increasing the supply of free cholesterol as a substrate and inducer of cholesterol 7α-hydroxylase resulting in the enhanced production of bile acids. In the rat *in vivo* under conditions of sufficient cholesterol supply (HFC/0.5), avasimibe treatment counteracted the effect of cholate-induced suppression and highly increased the expression of cholesterol 7α-hydroxylase.

TABLE 5. Effect of Avasimibe on Oxysterol 7α-Hydroxylase Activity

Diet	LFC (pmol/mg protein/hr)	HFC/0 (pmol/mg protein/hr)	HFC/0.5 (pmol/mg protein/hr)
Control	87 ± 11	177 ± 54	158 ± 26
+Avasimibe	66 ± 4 (76)	147 ± 25 (83)	213 ± 32 (135)

NOTE. Oxysterol 7α-hydroxylase activity in liver microsomes was measured in rats fed on various diets and treated with or without 0.01% (wt/wt) avasimibe. Data shown are means ± SEM (n = 4 per group). Values between parentheses represent data expressed as a percentage of value obtained in untreated animals on the same diet.

Incubation of hepatocytes with avasimibe resulted in a marked decrease in cellular cholesteryl ester content without a concomitant increase in free cholesterol levels. There are several explanations for this. Most importantly, the amount of free cholesterol becoming available after ACAT inhibition in the hepatocytes is rapidly diverted into the bile acid synthetic pathway, as was shown by this study. Furthermore, the high amount of free cholesterol in the cell membranes[38] probably overshadows changes in the free cholesterol content caused by the inhibition of ACAT. However, even small increases in the regulatory cholesterol pool, which is small when compared with the total free intracellular cholesterol mass,[31,38] can be monitored by measuring the suppression of the expression of genes involved in cholesterol synthesis and LDL-receptor–mediated uptake.[39,40] A decrease in the mRNA levels of HMG-CoA synthase and LDL-receptor was observed in cultured hepatocytes after incubation with avasimibe, indicating that the regulatory pool of free cholesterol is indeed enhanced. This is in agreement with previous studies in the human hepatoma cell line HepG2 using the ACAT inhibitor 58-035, showing a marked decrease in LDL-receptor activity.[31] In rats fed different diets, the decreased LDL-receptor mRNA levels will contribute to dietary cholesterol-induced increases in plasma cholesterol levels. However, treatment with avasimibe did not further induce LDL-receptor mRNA down-regulation, indicating that there is no further increase in the amount of regulatory cholesterol in the liver.

Other ACAT inhibitors have also been reported to influence bile acid synthesis. The ACAT inhibitor 58-034, DuP-128, and HL-004 showed an increase in bile acid synthesis in rat and hamster hepatocytes and in HepG2 cell, respectively.[41-43] In our study, we investigated the biochemical background to the induction of bile acid synthesis by the

ACAT inhibitor avasimibe and showed that this is owing to an increased expression of cholesterol 7α-hydroxylase both in control cells as well as in cells with a higher cholesterol content owing to the addition of βVLDL. Remarkably, avasimibe even enhanced cholesterol 7α-hydroxylase expression significantly above the level induced by βVLDL. The increased pool of regulatory cholesterol by incubation with avasimibe or βVLDL did not affect the expression of sterol 27-hydroxylase in these cells. Even in cells that already had an enhanced cholesterol content through the addition of βVLDL, avasimibe did not evoke any effect on sterol 27-hydroxylase expression. This is in line with the *in vivo* experiments in rats fed different diets, in which we also did not observe any effect on sterol 27-hydroxylase on diet or avasimibe treatment. Thus, our study and those of others[10,44] indicate that sterol 27-hydroxylase *in vivo* in rat and in rat hepatocytes is insensitive to cholesterol induction in contrast to the results from experiments with rabbits.[11] In addition, oxysterol 7α-hydroxylase was also not affected by ACAT inhibition in the rat fed different diets, indicating that this enzyme is insensitive to cholesterol, as was also observed in mice.[18] So, inhibition of ACAT only induces the primary route to bile acid synthesis, whereas the alternative route is not affected. In contrast to Schwarz et al.[18] we found no decrease in enzyme activity after feeding of a cholic acid containing diet (HFC/0.5) as compared with the HFC/0 diet. The reason for this discrepancy is not known, but may be related to the diet composition or a species difference.

The increased diversion of cholesterol into the bile acid synthetic pathway by the inhibition of hepatic ACAT resulted in a decline in the secretion of cholesteryl esters by the cells, which was accompanied by a decreased excretion of triglycerides. This reflects a suppressed VLDL-lipid excretion as also reported by others.[45,46]

In this study, we further evaluated the effects of avasimibe on cholesterol 7α-hydroxylase expression in the rat under various dietary conditions to increase plasma cholesterol and triglyceride levels. To that end, we used semisynthetic casein, sucrose-based diets containing different amounts of saturated fat and cholesterol. In these animals avasimibe proved to be a powerful cholesterol-lowering agent, in line with other studies with several rodent models[3] and in cynomolgus monkeys.[47] Avasimibe reduced total plasma cholesterol levels in both cholesterol-fed rat models and rats fed a cholesterol-free diet. Even in cholesterol-fed rats in which the plasma cholesterol levels were boosted by dietary cholate, avasimibe lowered the total plasma cholesterol levels below levels obtained by control cholesterol-free diet. An increase in non-HDL cholesterol and a decrease in HDL cholesterol characterize hypercholesterolemia in these studies. Avasimibe reduced non-HDL cholesterol under all dietary conditions. In the rats fed the control and cholesterol-rich diets no increase in HDL cholesterol was found with avasimibe. However, on the cholesterol-rich diet containing cholate, avasimibe enhanced HDL cholesterol, like other ACAT inhibitors that combine a decrease in non-HDL cholesterol with an increase in HDL cholesterol.[2,48] The cholesterol-fed rats also showed hypertriglyceridemia, which is prevented by avasimibe in agreement with the hepatocyte experiments, in which concomitantly hepatic secretion of cholesteryl esters and triglycerides was decreased. Increases in plasma triglyceride levels in cholesterol-fed rats (HFC/0 diet), counteracted by avasimibe treatment, reflected the accumulation of liver triglycerides, which are known to be related to the mass production and secretion of VLDL-triglyceride.[49-52]

The rat is able to adapt to large fluctuations in sterol input, because of a high basal level of cholesterol 7α-hydroxylase expression sensitive to induction by dietary cholesterol.[20,26] Treatment of rats on the cholesterol-free control diet (LFC) with avasimibe did not affect cholesterol 7α-hydroxylase expression, indicating that the amount of regulatory cholesterol available after ACAT inhibition in these rats is too low to evoke effects on cholesterol 7α-hydroxylase. Rats on the cholesterol-rich diet (HFC/0) did have a higher amount of cholesterol in the liver because hepatic cholesteryl esters were strongly increased as compared with the control diet (LFC). This high amount of hepatic cholesterol is an inducer for cholesterol 7α-hydroxylase[20,23,26,53,54] and a substrate for bile acid synthesis.[20,21,55] Treatment of rats on the cholesterol-rich diet with avasimibe did not further increase cholesterol 7α-hydroxylase enzyme activity and mRNA levels on the HFC/0 diet, indicating that the level of cholesterol 7α-hydroxylase expression cannot be further enhanced above the level induced on this diet.

Similarly, the overall process of bile acid synthesis was not further increased by avasimibe. This is probably because of the high basal level of bile acid synthesis on this diet, which was 3-fold increased compared with diet containing no cholesterol.[56] However, inhibition of hepatic ACAT may still contribute to the maintenance of this high basal level under conditions of a reduced transport of cholesterol to the liver by inhibition of intestinal ACAT. In addition, we found that there was no increase in the biliary excretion of cholesterol, indicating that ACAT inhibition does not result in a more lithogenic bile.

In the case of dietary cholate (HFC/0.5) the amount of cholesterol taken up by the intestine is increased and hepatic bile acid synthesis is suppressed, which is reflected by an increase in hepatic cholesteryl esters and also by an increase in liver free cholesterol levels. Thus, the amount of substrate and regulatory cholesterol required to induce cholesterol 7α-hydroxylase is high, but the situation is compromised by cholate down-regulating bile acid synthesis and cholesterol 7α-hydroxylase expression,[13,20,28,53,54] thereby interfering with the enzyme induction of cholesterol 7α-hydroxylase by cholesterol. Treatment with avasimibe reversed this condition by changing the balance between suppression and stimulation in favor of the up-regulation of cholesterol 7α-hydroxylase. There are several mechanisms that may contribute to the enhancing effect of avasimibe on cholesterol 7α-hydroxylase. The increasing amount of intestinal cholesterol, not absorbed because of the inhibition of intestinal ACAT, may interact with bile acids leading to malabsorption and a reduced potential of bile acid–induced feedback as suggested by Björkhem et al.[57,58] Further, the increase in the amount of regulatory cholesterol in the liver counteracts the effect of suppression of cholesterol 7α-hydroxylase expression by cholate. Otherwise, avasimibe may by itself reduce cholate uptake in the intestine.

In conclusion, we found that in cultured rat hepatocytes avasimibe increased bile acid synthesis by enhancing the supply of free cholesterol both as a substrate and inducer of cholesterol 7α-hydroxylase. Proper disposal of cholesterol into the bile acid synthetic pathway may contribute to the potent lipid-lowering effects of avasimibe in the rat. However, the primary hypocholesterolemic effect of avasimibe is caused

by its decreasing effect on cholesterol absorption by the inhibition of intestinal ACAT, thereby reducing the transport of cholesterol to the liver.

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