Inhibition of both the apical sodium-dependent bile acid transporter and HMG-CoA reductase markedly enhances the clearance of LDL apoB

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Abstract Discovery of the ileal apical sodium-dependent bile acid transporter (ASBT) permitted development of specific inhibitors of bile acid reabsorption, potentially a new class of cholesterol-lowering agents. In the present study, we tested the hypothesis that combining the novel ASBT inhibitor, SC-435, with the HMG-CoA reductase inhibitor, atorvastatin, would potentiate reductions in LDL cholesterol (LDL-C) and LDL apolipoprotein B (apoB). ApoB kinetic studies were performed in miniature pigs fed a typical human diet and treated with the combination of SC-435 (5 mg/kg/day) plus atorvastatin (3 mg/kg/day) (SC-435+A) or a placebo. SC-435+A decreased plasma total cholesterol by 23% and LDL-C by 40%. Multicompartmental analysis (SAAM II) demonstrated that LDL apoB significantly decreased by 35% due primarily to a 45% increase in the LDL apoB fractional catabolic rate (FCR). SC-435+A significantly decreased hepatic concentrations of free cholesterol and cholesteryl ester, and increased hepatic LDL receptor mRNA consequent to increased cholesterol 7α-hydroxylase expression and activity. In comparison, SC-435 (10 mg/kg/day) monotherapy decreased LDL apoB by 10% due entirely to an 18% increase in LDL apoB FCR, whereas atorvastatin monotherapy (3 mg/kg/day) decreased LDL apoB by 30% due primarily to a 22% reduction in LDL apoB production. We conclude that SC-435+A potentiates the reduction of LDL-C and LDL apoB due to complementary mechanisms of action.—Telford, D. E., J. Y. Edwards, S. M. Lipson, B. Sutherland, P. H. R. Barrett, J. R. Burnett, E. S. Krul, B. T. Keller, and M. W. Huff. **Inhibition of both the apical sodium-dependent bile acid transporter and HMG-CoA reductase markedly enhances the clearance of LDL apoB. *J. Lipid Res.* 2003.** 44: **943–952.**

Supplementary key words bile acid reabsorption • atorvastatin • kinetics • cholesterol 7α-hydroxylase • LDL receptor

Manuscript received 23 December 2002 and in revised form 30 January 2003.

Published, JLR Papers in Press, February 1, 2003.
DOI 10.1194/jlr.M200482-JLR200

Cloning of the ileal apical sodium-dependent bile acid transporter (ASBT) has identified a new pharmacologic target for modulation of plasma lipoproteins (1–4). SC-435 is a potent specific inhibitor of the ASBT, resulting in interruption of the enterohepatic circulation of bile acids and ultimately leading to decreased LDL cholesterol (LDL-C) in a variety of animal models (5–7). In vitro, SC-435 inhibits uptake of bile acids in BHK cells transfected with the human ASBT gene with an IC_{50} of 1.5 nM (6). In vivo, we recently demonstrated that, in miniature pigs fed normal amounts of fat and cholesterol, SC-435 enhanced bile acid excretion, leading to a significant 20% reduction in LDL-C (5). Apolipoprotein B (apoB) kinetic studies revealed that SC-435 treatment increased LDL apoB clearance, mediated by enhanced hepatic expression of LDL receptors secondary to upregulation of hepatic cholesterol 7α-hydroxylase (*CYP7A1*) expression. These effects are consistent with the molecular basis for *CYP7A1* regulation, recently elucidated by Lu et al. (8). Depletion of bile acids due to a decrease in their return to the liver reduces the farnesoid X receptor (FXR)-mediated activation of the short heterodimer partner (SHP) gene. Reduction in SHP allows for the induction of the liver receptor homolog-1 (LRH-1). LRH-1 is a positive transacting factor for the mouse ASBT gene (*slc10a2*) (9) and the *CYP7A1* gene (10, 11). Cholesterol 7α-hydroxylase, the product of *CYP7A1*, is the rate-limiting enzyme for the conversion of cholesterol to bile acids [as reviewed in ref. (12)]. An in-

Abbreviations: ASBT, apical sodium-dependent bile acid transporter; FCR, fractional catabolic rate; FXR, farnesoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LRH-1, liver receptor homolog-1; SHP, short heterodimer partner; SREBP, sterol regulatory element binding protein.

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crease in cholesterol 7α-hydroxylase activity results in a decrease in its substrate, hepatic cholesterol. This results in the activation of several compensatory reactions to maintain cellular cholesterol homeostasis, including upregulation of LDL receptor expression (5). We observed that SC-435 also increased the hepatic expression of HMG-CoA reductase and hence cholesterol biosynthesis, thereby attenuating the reduction in LDL-C (5).

In large clinical trials, reduction of cholesterol synthesis by statins, inhibitors of HMG-CoA reductase (the rate-limiting enzyme in cholesterol biosynthesis), effectively lowers LDL-C and cardiovascular endpoints (13–17). In vivo, apoB kinetic studies have demonstrated that statins enhance LDL receptor-mediated clearance of apoB-containing lipoproteins, and the ability of statins to reduce apoB production rates is also a consistent finding [as reviewed in ref. (18)]. Combination therapy with statins and bile acid sequestrants has been shown to be more effective for LDL-C reduction in human hyperlipidemic subjects and experimental animals than either treatment alone (19–22). This implies that combination of the ASBT inhibitor SC-435 and a statin would significantly enhance the reduction in plasma LDL-C and apoB through complementary mechanisms of action.

In the present study, we tested in miniature pigs the hypothesis that blocking the compensatory upregulation of hepatic cholesterol synthesis with statins potentiates the lowering of LDL-C by SC-435. Furthermore, apoB kinetic studies were carried out to establish the mechanism responsible for changes in lipoprotein metabolism. We found that the combination of SC-435 and atorvastatin significantly enhanced LDL-C reductions over either drug used in monotherapy. LDL apoB concentrations were significantly decreased primarily due to an increase in the LDL apoB fractional catabolic rate (FCR). The increase in VLDL apoB production rate observed with SC-435 alone was completely prevented by combination therapy. SC-435 plus atorvastatin significantly increased hepatic expression of cholesterol 7α-hydroxylase, resulting in decreased hepatic free cholesterol and cholesteryl ester, and consequently increased hepatic expression of the LDL receptor.

METHODS

Animals and diets

Miniature pigs weighing 28.3 ± 0.65 kg were obtained from Pureline Swine, Guelph, Ontario and were studied in pairs, each pair being same-sex littermates. After acclimatization for 1 week, animals were maintained on the experimental diet for 1 week prior to randomization to receive a placebo or the ASBT inhibitor, SC-435, at a dose of 5 mg/kg/day combined with atorvastatin (Pfizer Corporation) at a dose of 3 mg/kg/day. SC-435 is a benzothiepine-based small molecule inhibitor [1-[4-[4-[(4*R*,5*R*)-3,3-dibutyl-7-(dimethylamino)-2,3,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-benzothiepin-5-yl]phenoxy]butyl]-4-aza-1-azoniabicyclo[2.2.2]octane methanesulfonate (salt)] that was provided by Pharmacia Corp., St. Louis, MO. The specificity of SC-435 for ASBT has been described previously (5).

After 21 days of treatment, apoB kinetic studies were carried out over the subsequent 6 days. One week prior to each turnover study, an indwelling silicone elastomer (Silastic) catheter (1.96 mm internal diameter) was surgically implanted in an external jugular vein (21). Isoflurane USP (Abbott Laboratories Ltd.) was used as the anesthetic, and ketamine USP (Vetrepharm Canada Inc.) was used as the preanesthetic. Catheters, kept patent by filling with 7% EDTA-Na_2, allowed for ease of sample injection as well as blood sampling throughout each turnover study in unrestrained, unanesthetized animals (23). The experimental protocol was approved by the Animal Care Committee of the University of Western Ontario.

Either SC-435 plus atorvastatin or a placebo compound was placed in gelatin capsules and, to ensure ingestion, was administered by hand immediately prior to the daily feeding at 9 AM. Each animal received a 590 g ration of a diet containing fat (34% of calories; polyunsaturated-monounsaturated-saturated fatty acid ratio of 1:1:1; v/v/v) and cholesterol (400 mg/day; 0.1%; 0.2 mg/kcal) (23). Twenty-four hours after the last dose of SC-435 plus atorvastatin (9 AM), pigs were sacrificed and samples of the liver and intestine were rapidly frozen in liquid nitrogen.

Lipoprotein turnover studies

Lipoprotein turnover studies were performed essentially as described previously, with minor modifications (23, 24). In brief, VLDL ($d < 1.006$ g/ml) and LDL ($d = 1.019$–1.063 g/ml) from plasma (100–150 ml) obtained after a 24 h fast were isolated by sequential ultracentrifugation and subsequently radiolabeled with ^{131}I and ^{125}I, respectively (21). All labeled lipoproteins were autologous. Of the total VLDL radioactivity, <2% was free iodine, 10–38% was bound to lipid, and 35–54% of the protein-bound label was bound to apoB. Of the total LDL radioactivity, <1% was free iodine, 10–16% was bound to lipid, and 84–90% of the protein-bound label was bound to apoB. After a 24 h fast, each animal received 20 μCi ^{131}I-VLDL apoB, 15 μCi ^{125}I-LDL apoB, and 2.5 mCi L-[4,5-^3H]leucine (Amersham Canada Ltd., Oakville, Ontario; specific activity 155 Ci/mmol) given as a bolus by the indwelling catheter. Blood sample collection, administration of diet and drugs during the turnover study, lipoprotein isolation, and plasma apoB and plasma leucine determinations were as described previously (23). VLDL, IDL, and LDL apoB concentrations were constant over the sampling time period.

Kinetic analysis

The turnover data were analyzed using the multicompartmental modeling program SAAM II (SAAM Institute, Seattle, WA) running on a Pentium-based personal computer. The model structure, the assumptions made in developing the model, and the constraints applied to the model were essentially those previously reported (23, 25). Briefly, the compartmental model provided for the simultaneous fit of the ^{131}I, ^{125}I, and ^3H apoB tracer data. Because of the different methods by which the three tracers were introduced into the system, the information contained in each data set helped support different aspects of the model structure. The system model was constructed using the SAAM II program and included compartments for plasma leucine, apoB synthesis, and VLDL, IDL, and LDL apoB, as described previously (23). Three tracer experiments (^{131}I, ^{125}I, and ^3H) were created using the system model to specify the input and output (sample) sites of each tracer. Implicit in this approach is the equality of rate constants for a given pathway between all tracers. The different model compartments, their kinetic characteristics and rate constant constraints, and the initial distribution of ^{131}I-VLDL radioactivity across the lipoprotein fractions have been described previously (23). This model was simultaneously fit to the sets of tracer data for all lipoprotein fractions. Within the VLDL fraction, the model simultaneously described the ^3H and ^{131}I

VLDL tracer data; within the LDL fraction, the model described the kinetics of the ^{3}H, ^{131}I, and ^{125}I tracers. That the model could simultaneously describe data using different tracers provides support for the model structure and the use of different tracer-labeling methodologies for lipoprotein metabolic studies.

Hepatic total and microsomal lipids

Total liver lipids were extracted from 1.0 g sections of liver that had been obtained at sacrifice and stored at −80°C (5, 23). Microsomes were isolated from liver homogenates as described below and lipids were extracted from microsomes (1 mg protein) using the method of Folch et al. (26). Total cholesterol, free cholesterol, and triglyceride concentrations were quantitated in hepatic total and microsomal lipid extracts as described previously (23).

Microsome preparations

Microsomal fractions were prepared from liver homogenates by centrifugation at 10,000 *g* for 10 min at 4°C, followed by recentrifugation of the supernatant at 105,000 *g* for 2 h at 4°C, as described previously (5). Briefly, the pellet was resuspended and centrifuged at 400,000 *g* for 15 min at 4°C. The resulting pellet was then resuspended, homogenized, and aliquotted for storage at −80°C. The resulting hepatic microsomal fraction was used to measure cholesterol 7α-hydroxylase activity and HMG-CoA reductase activity.

Microsomal cholesterol 7α-hydroxylase activity and HMG-CoA reductase activity

Cholesterol 7α-hydroxylase activity was measured in hepatic microsomes using a modification (5) of a previously published method (27). Results are expressed as pmol of 7α-hydroxycholesterol formed/min/mg of microsomal protein. HMG-CoA reductase activity in hepatic microsomes was measured using a modification (5) of the method of Akerlund and Björkhem (28). Results are expressed as pmol of mevalonate formed/min/mg of microsomal protein.

Oleate incorporation into hepatic and intestinal cholesteryl ester and triglyceride

The synthesis of triglyceride and cholesteryl ester was determined in crude homogenates prepared from liver or intestinal samples stored at −80°C. The rate of incorporation of [1-^{14}C]oleoyl-CoA (Amersham) into cholesteryl ester and triglyceride was determined essentially as described by Krause et al. (29).

Measurement of mRNA abundance

Total RNA was isolated from liver using Trizol reagent (Life Technologies, Mississauga, Ontario). Custom oligonucleotide primers were generated corresponding to pig apoB (GenBank database accession # M20384.1) 5′-GGA GAG TCT AGG TTT TCT TTC AGG AGT CTA AAA CCC AAT GGG GC-3′; pig LDL receptor (accession # AF065990) 5′-GGG ATG CAG GTG GAG CTG TTG CAC TGG AAG CTG GCG GGG CCA CAG GTG GGT GTG GGG-3′; pig cholesterol 7α-hydroxylase (accession # AF020321.1) 5′-CGG ATG TTA TAG GAT CCG TCC TGG AGG TGC AAA GTG AAA TCC-3′; human HMG-CoA reductase (accession # XM003932.2) 5′-TCC CAG GGA TGG GAG GCC ACA AAG AGG CCA TGC AT-3′; and pig glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (accession # U82261.1) 5′-CGG AAG GCC ATG CCA GTG AGC TTC-3′. Oligonucleotides (5 pmol) were 5′-end labeled by incubating for 10 min at 37°C with [^{32}P]ATP (7,000 Ci/mmol, Amersham) and 10 U/μl T4 polynucleotide kinase (Life Technologies). They were then reisolated after filtering through quick-spin centrifuge columns (Boehringer Mannheim). Oligonucleotide probes for the gene of interest, together with the oligonucleotide probe for GAPDH, were hybridized to total RNA (20 μg) from either control or treated liver tissue and incubated for 10 min at 75°C and then overnight at 55°C (30). Following hybridization, samples were incubated for 30 min at 37°C with 300 units of S1 nuclease (Boehringer

TABLE 1. Plasma lipid and lipoprotein concentrations in control and SC-435 plus atorvastatin-treated miniature pigs

	Triglyceride		Cholesterol				ApoB	
	Total	VLDL	Total	VLDL	LDL	HDL	VLDL	LDL
	mmol/l						*mg/l*	
Control								
1	0.20	0.08	2.45	0.02	1.40	1.03	10.30	212.10
2	0.40	0.29	2.91	0.04	1.31	1.56	14.51	230.60
3	0.37	0.22	3.46	0.07	1.81	1.59	15.37	261.10
4	0.21	0.10	2.88	0.05	1.55	1.29	15.80	260.10
5	0.30	0.15	2.94	0.03	1.44	1.47	11.30	222.10
6	0.38	0.26	2.71	0.07	1.15	1.47	10.92	154.70
Mean	0.31	0.18	2.89	0.05	1.44	1.40	13.03	223.45
SEM	0.04	0.04	0.14	0.01	0.09	0.09	1.00	15.99
SC-435 + atorvastatin								
1	0.16	0.06	2.10	0.02	1.01	1.07	10.28	153.00
2	0.36	0.21	2.24	0.04	0.86	1.35	14.69	175.00
3	0.47	0.33	2.28	0.05	0.96	1.26	15.30	141.90
4	0.29	0.18	2.29	0.04	0.86	1.39	14.83	131.20
5	0.35	0.21	2.58	0.04	0.89	1.70	10.77	158.30
6	0.38	0.22	1.86	0.04	0.58	1.24	10.15	116.80
Mean	0.34	0.20	2.23	0.04	0.86	1.34	12.67	146.03
SEM	0.04	0.04	0.10	0.01	0.06	0.09	1.02	8.43
P	0.358	0.578	0.004	0.224	0.0004	0.493	0.111	0.004

Each lipid value is a mean of three determinations from each animal. VLDL cholesterol was determined after ultracentrifugation at d < 1.006 g/ml. LDL cholesterol was calculated as total cholesterol minus the sum of VLDL cholesterol and HDL cholesterol. HDL cholesterol was determined after precipitation of the apoB-containing lipoproteins from plasma. VLDL (d < 1.006 g/ml) and LDL (d = 1.019 to 1.063 g/ml) apoB are the mean of all samples obtained during the kinetics study in the respective lipoprotein fractions separated by ultracentrifugation.

Mannheim) and precipitated with ethanol. Probes were separated using denaturing polyacrylamide (19%) gel electrophoresis. Bands were visualized using a phosphorimager and quantitated using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Data are expressed as band intensity for the gene of interest compared with those obtained for GAPDH.

Analyses

Fasting blood samples (20 ml) were taken on days 1, 4, and 6 of the turnover study, and plasma concentrations of total cholesterol and triglyceride, VLDL-C and triglyceride, and HDL-C were measured (21). VLDL was obtained after ultracentrifugation at d < 1.006 g/ml, and HDL was obtained after precipitation of other lipoproteins by dextran sulfate-magnesium chloride (21). LDL-C was calculated by difference. The concentrations of total cholesterol, triglyceride, free cholesterol, cholesteryl ester, phospholipids, and protein were measured in the plasma and various lipoprotein fractions as described previously (21). Differences between control and combination treatment animals were compared for statistical significance by paired Student's *t*-test. Differences between monotherapy treatment and combination treatment animals were compared for statistical significance by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Animals received daily oral doses of SC-435 (5 mg/kg/day) and atorvastatin (3 mg/kg/day). The SC-435 dose was half that used previously in monotherapy (5), in which fecal bile acid excretion was increased 1.8-fold and LDL-C was reduced by 20%. Previously, we demonstrated that 3 mg/kg/day of atorvastatin results in a LDL-C reduction of 30% (23). Therefore, these doses were chosen for further study in order to achieve our objective, which was to determine if a low dose of SC-435 could potentiate the effect of a moderate dose of atorvastatin on plasma LDL-C concentrations and plasma apoB kinetics.

The effect of SC-435 combined with atorvastatin on plasma and lipoprotein lipid concentrations following 21 days of treatment is shown in **Table 1**. Combination treatment significantly reduced total plasma cholesterol by 23% ($P < 0.004$) and LDL-C by 40% ($P < 0.0004$). HDL-C was unchanged. Total plasma triglycerides, VLDL triglycerides, and VLDL apoB concentrations were unchanged, whereas LDL apoB decreased by 35% ($P < 0.004$).

Autologous ^{131}I-VLDL, ^{125}I-LDL, and [^{3}H]leucine were simultaneously injected into each control pig and each pig treated with SC-435 plus atorvastatin. The kinetic parameters of apoB were determined from the simultaneous analysis of all the specific activity data using the models described previously (23–25). The kinetic parameters for VLDL apoB and LDL apoB are summarized in **Tables 2** and **3**. A fit of the model to the apoB-specific radioactivity curves for VLDL, IDL, and LDL following the injection of tracers for a representative pair of animals is shown in **Fig. 1**.

Combination treatment had little or no effect on the VLDL apoB kinetic parameters. VLDL apoB pool size, FCR, and production rates were unchanged. Neither the amount of VLDL apoB converted to LDL apoB nor the flux of apoB cleared directly without conversion to IDL or LDL was affected by combination treatment. The percent-

TABLE 2. Metabolic parameters of VLDL apoB metabolism in control and SC-435 plus atorvastatin-treated miniature pigs

	VLDL Pool Size[a]	VLDL FCR[b]	VLDL Total Production	VLDL Conversion to LDL		VLDL Direct Removal	
	mg/kg	h^{-1}	$mg \cdot kg^{-1} \cdot h^{-1}$	$mg \cdot kg^{-1} \cdot h^{-1}$	%[c]	$mg \cdot kg^{-1} \cdot h^{-1}$	%[d]
Control							
1	0.46	4.43	2.06	0.12	6	1.64	80
2	0.65	4.91	3.21	0.12	4	3.09	96
3	0.69	5.00	3.46	0.10	3	3.30	95
4	0.71	4.00	2.85	0.12	4	2.57	90
5	0.51	4.52	2.30	0.14	6	2.15	93
6	0.49	4.15	2.04	0.07	3	1.91	94
Mean	0.59	4.50	2.65	0.11	4	2.44	91
SEM	0.05	0.16	0.25	0.01	1	0.27	3
SC-435 + atorvastatin							
1	0.46	2.77	1.28	0.20	16	1.08	84
2	0.66	3.54	2.34	0.21	9	2.14	91
3	0.69	5.25	3.61	0.13	4	3.45	96
4	0.67	4.99	3.33	0.11	3	3.20	96
5	0.49	5.71	2.80	0.08	3	2.71	97
6	0.46	4.42	2.02	0.10	5	1.92	95
Mean	0.57	4.45	2.56	0.14	7	2.42	93
SEM	0.05	0.45	0.35	0.02	2	0.36	2
P	0.158	0.915	0.730	0.282	0.298	0.921	0.305

FCR, fractional catabolic rate. Kinetic parameters were determined by using SAAM II.

[a] Pool size refers to the plasma VLDL apoB concentration multiplied by 0.042, with the assumption that in the pig there are 42 ml plasma per kilogram body weight.

[b] FCR is determined by [FLUX(0,1) + FLUX(2,1) + FLUX(4,1) + FLUX(6,1)]/VLDL apoB pool size, where FLUX (0,1)+FLUX(2,1)+FLUX(4,1)+FLUX(6,1) is the production rate of VLDL apoB, where the numbers in brackets refer to the compartments of the model.

[c] Values represent VLDL conversion to LDL as a percent of total VLDL production.

[d] Values represent VLDL direct removal as a percent of total VLDL production.

TABLE 3. Metabolic parameters of LDL apoB metabolism in control and SC-435 plus atorvastatin-treated miniature pigs

	LDL Pool Size[a]	LDL FCR[b]	LDL Total Production	LDL Direct Production[c]		Total apoB Production[e]
	mg/kg	h^{-1}	$mg \cdot kg^{-1} \cdot h^{-1}$	$m \cdot kg^{-1} \cdot h^{-1}$	%[d]	$mg \cdot kg^{-1} \cdot h^{-1}$
Control						
1	9.54	0.04	0.34	0.30	88	2.35
2	10.38	0.05	0.47	0.35	74	3.55
3	11.75	0.03	0.36	0.27	75	3.73
4	11.70	0.04	0.48	0.36	75	3.21
5	10.00	0.05	0.48	0.33	70	2.63
6	6.96	0.04	0.29	0.22	76	2.25
Mean	10.06	0.04	0.40	0.31	76	2.95
SEM	0.72	0.002	0.03	0.02	3	0.26
SC-435 + atorvastatin						
1	6.89	0.08	0.52	0.31	60	1.59
2	7.88	0.07	0.54	0.33	61	2.67
3	6.38	0.06	0.36	0.23	64	3.84
4	5.90	0.06	0.34	0.23	68	3.56
5	7.21	0.05	0.33	0.25	75	3.05
6	5.25	0.05	0.26	0.16	62	2.18
Mean	6.59	0.06	0.39	0.25	65	2.82
SEM	0.39	0.004	0.05	0.03	2	0.35
P	0.004	0.018	0.830	0.044	0.047	0.570

Kinetic parameters were determined by using SAAM II.

[a] Pool size refers to the plasma LDL apoB concentration multiplied by 0.042, with the assumption that in the pig there are 42 ml plasma per kilogram body weight.

[b] FCR is the rate constant k(0,6) determined from the model.

[c] Direct production (direct synthesis) is the production of apoB directly into compartment 6.

[d] LDL direct production as a percent of total LDL production.

[e] Production of apoB into the plasma compartment calculated as VLDL apoB production plus LDL apoB direct production.

age of VLDL apoB converted to LDL apoB and the percentage cleared directly were unaffected by SC-435 plus atorvastatin. In addition, IDL apoB kinetic parameters were not changed by treatment (data not shown).

SC-435 plus atorvastatin treatment significantly decreased the LDL apoB pool size by 35% ($P < 0.004$) (Table 3). This was primarily due to a 45% ($P < 0.018$) increase in the LDL apoB FCR. Combination treatment had no effect on the total production rate of LDL apoB or on the amount of LDL apoB derived from VLDL apoB catabolism. However, LDL apoB direct synthesis, defined as LDL apoB entering the plasma compartment without initial metabolism through the plasma VLDL or IDL pools, was decreased by 18%, although this change was of borderline statistical significance ($P < 0.044$). Total production of apoB into plasma (VLDL plus LDL direct synthesis) decreased by 5%, but this change was not statistically significant.

The kinetic parameters calculated for apoB transport into plasma and through the metabolic cascade in pigs treated with SC-435 and atorvastatin as compared with controls are illustrated in **Fig. 2**. VLDL apoB kinetics were unchanged by combination therapy. The LDL apoB pool was significantly decreased, primarily due to an increase in the LDL apoB FCR, although LDL apoB direct synthesis was also significantly reduced. Examination of Fig. 2B allows for a comparison of apoB kinetics in pigs treated with SC-435 plus atorvastatin, and pigs treated with SC-435 alone. The latter experiment was recently published and was performed using an identical protocol to that of the present study (5). The increase in VLDL apoB production observed with SC-435 alone was completely prevented by combination treatment (22 ± 7% vs. −3 ± 0.2%, $P < 0.04$). As a result, the VLDL apoB pool size increased with SC-435 alone, whereas VLDL apoB pool size was unchanged (17 ± 7% vs. −3% ± 1%, $P < 0.04$) with combination treatment. Combination treatment decreased the LDL apoB pool size to a greater extent than that observed with SC-435 alone (−35 ± 6% vs. −10 ± 4%, $P < 0.003$), and the LDL apoB FCR increased to a greater extent with combination treatment as compared with SC-435 alone (45 ± 16% vs. 17 ± 6%, $P < 0.05$).

VLDL and LDL were analyzed for lipid and protein composition as shown in **Table 4**. SC-435 in combination with atorvastatin resulted in a 63% decrease ($P < 0.01$) in the percentage of cholesteryl ester in VLDL. The percent composition of other VLDL lipids and protein were not significantly different. Combination treatment increased the percentage of triglyceride in LDL by 102% ($P < 0.001$), decreased the percentage of cholesteryl ester by 13% ($P < 0.001$), and increased the percentage of protein by 9% ($P < 0.033$). The percent composition of other lipids was unchanged. The ratio of triglyceride-cholesteryl ester (wt/wt) in LDL increased by 135% ($P < 0.002$). The ratio of surface to core lipids was unchanged, suggesting that combination therapy did not alter LDL particle size.

Approximately 24 h after the last dose of the drugs was administered, pigs were sacrificed and sections of liver and small intestine were removed and stored at −80°C prior to analyses. Hepatic concentrations of free choles-

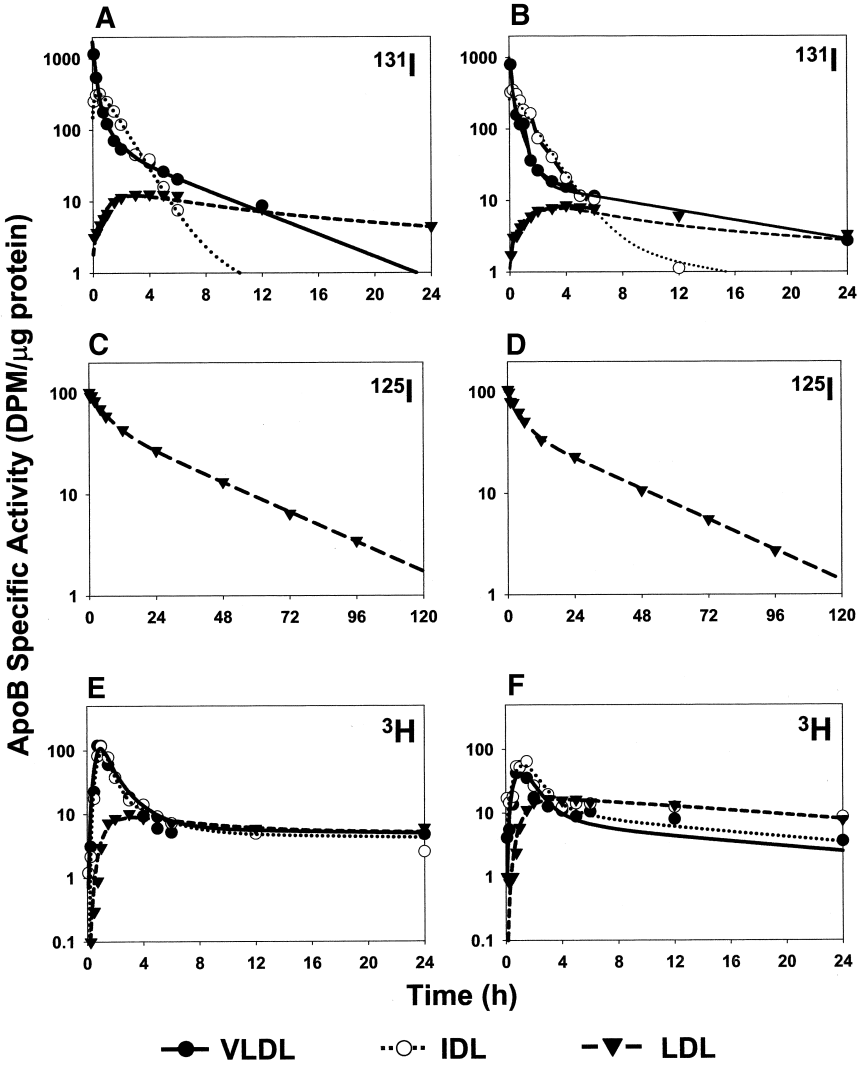

Fig. 1. Representative apolipoprotein B (apoB)-specific activity-time curves for VLDL, IDL, and LDL after the injection of ^{131}I-VLDL (A and B), ^{125}I-LDL (C and D), or [^{3}H]leucine (E and F). Data points represent the observed data, and the lines are the best fit generated by the kinetic model using SAAM II. A, C, E: Control; B, D, F: A pig treated with SC-435 plus atorvastatin.

terol and cholesteryl ester were decreased by 12% ($P < 0.049$) and 47% ($P < 0.026$), respectively (**Table 5**). Liver triglycerides decreased by 8%, but this change was not statistically significant. Reductions in hepatic microsomal free cholesterol (−13%), cholesteryl ester (−24%), and triglyceride (−11%) were observed; however, only the cholesteryl ester reduction was significant ($P < 0.048$). Intestinal lipid concentrations were not affected by combination treatment (data not shown). The activity of cholesterol 7α-hydroxylase was increased 2.2-fold ($P < 0.033$) in hepatic microsomes by SC-435 plus atorvastatin as compared with a placebo (Table 5). Furthermore, the activity of HMG-CoA reductase was also increased 10-fold ($P < 0.019$) in the livers of treated animals. The incorporation of oleate into cholesteryl ester and triglyceride in the intestine were also unchanged by treatment (data not shown).

The liver concentrations of mRNA for the LDL receptor, cholesterol 7α-hydroxylase, and HMG-CoA reductase were increased by combination treatment (Table 5). The mRNA abundance of the LDL receptor increased 1.4-fold ($P < 0.01$), of cholesterol 7α-hydroxylase increased 3.4-fold ($P < 0.038$), and of HMG-CoA reductase increased 2.5-fold ($P < 0.013$). Hepatic apoB mRNA was unchanged (data not shown).

Figure 3 summarizes the major lipid and apoB kinetic parameters observed in this study for treatment with SC-435 (5 mg/kg/day) plus atorvastatin (3 mg/kg/day). For comparison purposes, parameters obtained previously in similar studies using SC-435 monotherapy (10 mg/kg/day) (5), atorvastatin monotherapy (3 mg/kg/day) (23), or atorvastatin monotherapy (10 mg/kg/day) (24) are also shown in Fig. 3. In general, reductions in LDL-C and

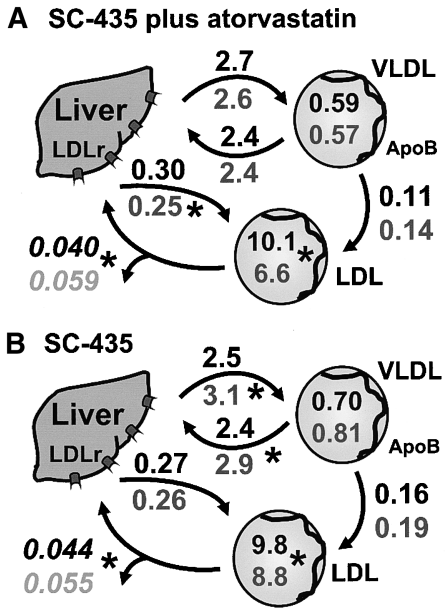

Fig. 2. Diagram summarizing the effects of (A) SC-435 (5 mg/kg/day) plus atorvastatin (3 mg/kg/day) treatment (gray shading), compared with control (black) and (B) SC-435 (10 mg/kg/day) monotherapy (gray shading) compared with the control (black) on apoB metabolism. Kinetic parameters were calculated using SAAM II. Circled numbers represent mean apoB pool sizes in mg/kg, numbers next to arrows represent mean transport rates (production rates) in mg/kg/h, and numbers in italics next to arrows represent fractional catabolic rates in pools/h. * $P < 0.05$ versus control.

LDL apoB concentrations with combination therapy were greater than those observed with treatments of SC-435 alone or atorvastatin (3 mg/kg/day) alone. Changes in these parameters were similar to those obtained with 10 mg/kg/day atorvastatin. Reductions in total LDL apoB production rates with combination therapy were less than those observed with either dose of atorvastatin, but greater than those observed with SC-435 alone. However, the increase in LDL apoB FCR with combination treatment was ~3-fold greater than that observed with monotherapy with either SC-435 or atorvastatin (both doses), suggesting a synergistic effect with respect to LDL apoB clearance.

TABLE 4. Percent composition of VLDL and LDL isolated from control and SC-435 plus atorvastatin-treated miniature pigs

	VLDL			LDL		
	Control	SC-435 and Atorvastatin	*P*	Control	SC-435 and Atorvastatin	*P*
Triglyceride	64.93	73.36	0.129	3.66	7.40	0.001
Free cholesterol	5.72	4.83	0.372	9.24	8.77	0.148
Cholesteryl ester	4.82	1.80	0.010	33.36	29.09	0.001
Phospholipid	13.91	10.86	0.095	26.76	25.41	0.059
Protein	10.63	9.14	0.573	26.99	29.34	0.033
TG/CE[a]	14.42	22.33	0.681	0.11	0.26	0.002
Surface/core[b]	0.29	0.21	0.151	0.97	0.94	0.069

Values are percent of total lipoprotein and are means of determinations on VLDL and LDL from six control and six SC-435 plus atorvastatin-treated pigs.

[a] Ratios are weight ratios of triglyceride to cholesteryl ester.

[b] Surface/core is the ratio of (phospholipid+free cholesterol)/(cholesteryl ester+triglyceride).

TABLE 5. Hepatic lipids, enzyme activities, and mRNA expression in control and SC-435 plus atorvastatin-treated miniature pigs

	Control	SC-435 and Atorvastatin	n	*P*
Lipid; total, mg/g wet weight				
Free cholesterol	2.32 ± 0.08	2.04 ± 0.12	6	0.049
Cholesteryl ester	0.35 ± 0.03	0.18 ± 0.05	6	0.026
Triglyceride	2.30 ± 0.09	2.11 ± 0.17	6	0.338
Lipids; microsomal, μg/mg protein				
Free cholesterol	84 ± 5	73 ± 4	6	0.115
Cholesteryl ester	8.5 ± 0.8	6.5 ± 1.0	6	0.048
Triglyceride	85 ± 8	75 ± 7	6	0.284
Enzyme activities, pmol · min^{-1} · mg^{-1}				
Cholesterol 7α-hydroxylase	70.8 ± 17.2	158.6 ± 37.3	6	0.033
HMG-CoA reductase	3.4 ± 0.5	34.7 ± 11.1	6	0.019
Oleate incorporation				
Cholesteryl ester	356 ± 37	442 ± 26	6	0.091
Triglyceride	271 ± 12	229 ± 14	6	0.060
mRNA expression,[a] percent of control				
Cholesterol 7α-hydroxylase	100 ± 21	335 ± 99	6	0.038
LDL receptor	100 ± 6	133 ± 9	5	0.010
HMG-CoA reductase	100 ± 11	254 ± 57	6	0.013

Values represent the mean ± SEM. *P* values are for comparisons between control and SC-435 plus atorvastatin treatment. Livers were removed ~24 h after the last dose of SC-435 plus atorvastatin or placebo. Sections from several lobes of liver were excised and frozen at −80°C until analysis. The activities of cholesterol 7α-hydroxylase and HMG-CoA reductase were determined in hepatic microsomes. Oleate incorporation into cholesteryl ester and triglycerides was determined in crude homogenates, and free cholesterol, cholesteryl ester, and triglyceride concentrations were determined in whole liver and hepatic microsomes by using enzymatic reagents as described in Methods.

[a] Total RNA was extracted from liver tissue and quantitated by RNase protection as described in Methods. Results are expressed as percent of control (ratio of band intensity relative to glyceraldehyde 3-phosphate dehydrogenase).

DISCUSSION

The discovery of ASBT and the elucidation of the complex series of events whereby FXR regulates the activity of cholesterol 7α-hydroxylase has refocused attention on reducing the enterohepatic circulation of bile acids as an alternate means for treatment of hypercholesterolemia (1–3, 8, 10, 11, 31). The ASBT has become an attractive target for therapeutic intervention due to its critical role in bile acid reabsorption and its localization to the lumenal surface of enterocytes within the terminal ileum. This has facilitated the development of specific ASBT inhibitors, which, despite the absence of systemic absorption, are able to regulate the metabolism of hepatic cholesterol, ultimately resulting in significant reductions in plasma LDL-C (6, 32–34). Recently, we reported that treatment of miniature pigs with the ASBT inhibitor SC-435 significantly decreased LDL-C (−20%) and apoB (−10%) (5). The primary mechanism for this effect was enhanced LDL apoB clearance, which was mediated by increased expression of hepatic LDL receptors, secondary to up-regulation

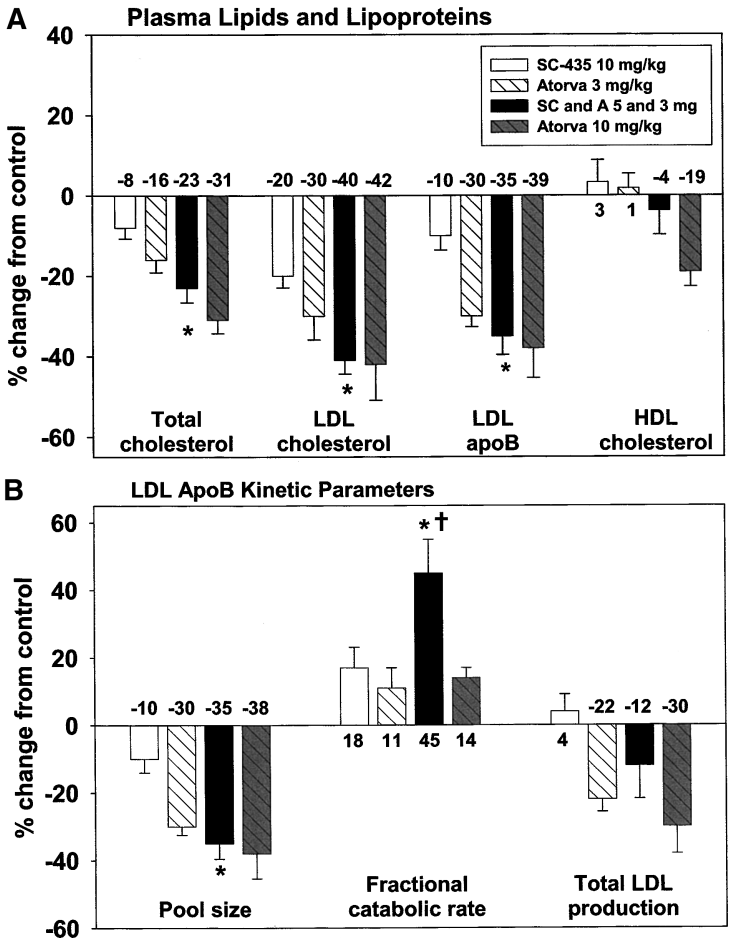

Fig. 3. Bar graphs showing the percent change from control in (A) plasma and lipoprotein lipids and (B) LDL apoB kinetic parameters in pigs treated with SC-435 monotherapy (10 mg/kg/day), atorvastatin monotherapy (3 mg/kg/day and 10 mg/kg/day), or the combination of SC-435 (5 mg/kg/day) plus atorvastatin (3 mg/kg/day). *Significantly different from SC-435 monotherapy (10 mg/kg/day). †Significantly different from atorvastatin monotherapy (3 mg/kg/day).

of hepatic cholesterol 7α-hydroxylase. A compensatory increase in hepatic HMG-CoA reductase expression and activity was observed, suggesting that the up-regulation of cholesterol biosynthesis in response to chronic inhibition of bile acid reabsorption might be responsible for the modest plasma LDL-C-lowering activity observed.

In the present study, we establish that inhibition of hepatic cholesterol synthesis with the HMG-CoA reductase inhibitor atorvastatin would block this compensatory increase in synthesis and result in a greater reduction in plasma cholesterol when combined with the ASBT inhibitor SC-435. The combination of SC-435 and atorvastatin caused a significantly enhanced reduction in LDL-C of 40%, 2-fold greater than SC-435 alone. Furthermore, LDL apoB was decreased by 35%, 3.4-fold over SC-435 alone. This marked reduction in LDL apoB was primarily due to a 45% increase in LDL apoB clearance, which was mediated by enhanced hepatic LDL receptor expression consequent to upregulation of hepatic cholesterol 7α-hydroxylase activity and expression and decreased cholesterol biosynthesis.

With respect to monotherapy, we observed that SC-435 inhibition of the ASBT in this animal model led to increased bile acid excretion and subsequent upregulation of cholesterol 7α-hydroxylase expression and activity (5). When SC-435 was combined with atorvastatin, cholesterol 7α-hydroxylase was activated to a similar extent. This was anticipated, since statin therapy alone would not be expected to influence the enterohepatic circulation of bile acids and/or cholesterol 7α-hydroxylase activity. Of significance, the inhibition of HMG-CoA reductase activity by atorvastatin resulted in an overexpression of HMG-CoA reductase at the level of both message and activity as a result of a positive-feedback regulatory mechanism (35, 36). As discussed previously, the extent to which HMG-CoA reductase activity and expression are upregulated in the presence of a statin is proportional to the magnitude of HMG-CoA reductase enzyme inhibition (24). These results indicate that the compensatory increase in hepatic cholesterol biosynthesis resulting from the SC-435-induced increase in cholesterol 7α-hydroxylase activity was blocked by the addition of atorvastatin.

These observations support our hypothesis that combination therapy enhances the reduction of a regulatory pool of hepatic microsomal cholesterol responsible for the regulation of LDL receptor expression. In whole liver, significant reductions in both free cholesterol and cholesteryl ester concentrations were observed, and in hepatic microsomes, cholesteryl ester concentrations were reduced and a trend toward a lower free cholesterol concentration was observed. These results are consistent with our current understanding of the regulation of hepatic cholesterol homeostasis at the molecular level (8, 10, 11). SC-435-induced inhibition of bile acid recycling to the liver

would reduce the FXR-mediated activation of the SHP gene. Reduced levels of SHP would reduce the SHP-mediated repression of LRH-1. LRH-1 binds to and is an activator of the cholesterol 7α-hydroxylase gene promoter. Therefore, removing its repressor, SHP, would increase the conversion of hepatic cholesterol to bile acids. A reduction in the hepatic regulatory pool of cholesterol would be enhanced by the HMG-CoA reductase inhibitor atorvastatin, leading to a further increase in LDL receptor expression. Although not measured in this study, it is likely that this response was mediated by an increase in the mature nuclear form of sterol regulatory element binding protein-2 (SREBP-2). SREBP-2 is a strong transcriptional activator of LDL receptor and HMG-CoA reductase expression (37), both of which were observed to increase in this study. It is less likely that increased LDL receptor expression was mediated by activation of SREBP-1, since the major effect of this transcription factor is activation of hepatic fatty acid and triglyceride synthesis (37), neither of which were affected by combination treatment. This interpretation is consistent with the results of Sheng et al. (38), who reported that, in hamsters, treatment with the bile acid sequestrant colestipol and the HMG-CoA reductase inhibitor mevinolin increased the hepatic nuclear form of SREBP-2, whereas that of SREBP-1 was decreased.

We might have anticipated that the reduction in hepatic cholesterol subsequent to the increased activity of cholesterol 7α-hydroxylase and inhibition of cholesterol synthesis would decrease the availability of cholesterol/cholesteryl ester for VLDL assembly (18). Our previous studies in pigs treated with atorvastatin alone demonstrated significant decreases in VLDL secretion directly related to reductions in hepatic cholesterol and cholesteryl ester concentrations (23, 24). However, combination treatment had no effect on VLDL apoB secretion. This finding also contrasts with our previous studies where SC-435 monotherapy in pigs increased VLDL secretion (5). We concluded that, while increased triglyceride production had been observed in patients treated with bile acid sequestrants (39), the effect we observed in SC-435-treated pigs was unrelated to changes in hepatic triglyceride metabolism, as neither triglyceride mass nor triglyceride synthesis were altered (5). The lack of change in VLDL secretion with combination therapy suggests that the mechanism responsible for the enhanced VLDL apoB secretion by SC-435 alone was blocked by the addition of atorvastatin.

LDL direct synthesis was reduced, making a small contribution to the decrease in LDL apoB concentration. This finding is consistent with our previous studies, in which direct LDL synthesis was inhibited in pigs treated with cholestyramine plus lovastatin (20, 21). However, we have not observed any changes in this parameter in pigs treated with atorvastatin monotherapy (23, 24). In the latter studies, LDL apoB was significantly reduced as a result of decreased LDL production consequent to decreased VLDL secretion, together with an increased LDL apoB FCR.

SC-435 plus atorvastatin significantly altered the composition of LDL; specifically, a decrease in cholesteryl ester and a trend to smaller particles. It is likely that this contributed to the substantial increase in LDL apoB FCR. Previously, changes in LDL composition associated with decreased cholesteryl ester content resulting in reduced LDL particle size have been associated with an increase in LDL apoB FCR in guinea pigs treated with either cholestyramine (40) or lovastatin (41).

We conclude that SC-435 plus atorvastatin potentiates the reduction of LDL-C and apoB through complementary mechanisms of action. In a large animal model of human lipoprotein metabolism, blocking bile acid reabsorption with a specific inhibitor of the ASBT and decreasing the compensatory increase in cholesterol biosynthesis with atorvastatin results in substantial reductions in LDL-C and LDL apoB. The primary mechanism is increased LDL clearance, which is mediated through enhanced hepatic expression of LDL receptors subsequent to a combination of increased hepatic cholesterol 7α-hydroxylase activity and decreased cholesterol synthesis. Thus, inhibition of the ileal ASBT represents a potential new treatment for hypercholesterolemia and, in combination with statins, provides an effective means for achieving even lower target lipid values.

This work was supported by a grant from the Heart and Stroke Foundation of Ontario (T-4386 to M.W.H.), the National Institutes of Health (NCRR Grant RR-12609), and Pharmacia Corporation. P.H.R.B. is a Career Development Fellow of the National Heart Foundation of Australia. M.W.H. is a Career Investigator of the Heart and Stroke Foundation of Ontario. The authors thank Kim Wood for performing the surgeries and Stefanie Bombardier and Nida Napawan for technical assistance.

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