The molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin

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Abstract The combination of ezetimibe, an inhibitor of Niemann-Pick C1-like 1 protein (NPC1L1), and an HMG-CoA reductase inhibitor decreases cholesterol absorption and synthesis. In clinical trials, ezetimibe plus simvastatin produces greater LDL-cholesterol reductions than does monotherapy. The molecular mechanism for this enhanced efficacy has not been defined. Apolipoprotein B-100 (apoB-100) kinetics were determined in miniature pigs treated with ezetimibe (0.1 mg/kg/day), ezetimibe plus simvastatin (10 mg/kg/day), or placebo (n = 7/group). Ezetimibe decreased cholesterol absorption (−79%) and plasma phytosterols (−91%), which were not affected further by simvastatin. Ezetimibe increased plasma lathosterol (+65%), which was prevented by addition of simvastatin. The combination decreased total cholesterol (−35%) and LDL-cholesterol (−47%). VLDL apoB pool size decreased 26%, due to a 35% decrease in VLDL apoB production. LDL apoB pool size decreased 34% due to an 81% increase in the fractional catabolic rate, both of which were significantly greater than monotherapy. Combination treatment decreased hepatic microsomal cholesterol (−29%) and cholesteryl ester (−65%) and increased LDL receptor (LDLR) expression by 240%. The combination increased NPC1L1 expression in liver and intestine, consistent with increased SREBP2 expression. Ezetimibe plus simvastatin decreases VLDL and LDL apoB-100 concentrations through reduced VLDL production and upregulation of LDLR-mediated LDL clearance.—Telford, D. E., B. G. Sutherland, J. Y. Edwards, J. D. Andrews, P. H. R. Barrett, and M. W. Huff. **The molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin. *J. Lipid Res.* 2007.** 48: **699–708.**

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Increased concentrations of LDL-cholesterol represent a major risk factor for atherosclerosis (1, 2). Clinical trials demonstrate that reduction of plasma LDL-cholesterol by diet and/or drugs constitutes a primary strategy for the prevention and regression of coronary heart disease (1, 2). LDL concentrations are regulated primarily in the liver; it is the source of VLDL, the precursor of plasma LDL, and is responsible for the majority of LDL clearance via the LDL receptor (LDLR) (3). Apolipoprotein B-100 (apo B-100) kinetic studies in patients with primary hypercholesterolemia and combined hyperlipidemia demonstrate that LDL-cholesterol concentrations are determined by the production and catabolism of LDL particles (4, 5). HMG-CoA reductase inhibitors (statins) decrease cholesterol synthesis primarily in the liver (3), resulting in reduced hepatocyte cholesterol concentrations and, ultimately, upregulation of the LDLR and enhanced clearance of LDL (as reviewed in Ref. 6). In some patients, statins decrease rates of VLDL production (7).

The liver also clears cholesterol absorbed from the small intestine, via uptake of chylomicron remnants (3, 8). In humans, approximately one third of cholesterol entering the small intestine is of dietary origin, whereas two thirds is derived from bile (9). Inhibition of cholesterol absorption as a therapeutic target has gained considerable attention with the introduction of ezetimibe, the first of a new class of selective cholesterol absorption inhibitors (10–13).

Recent research (14–16) has revealed that ezetimibe specifically inhibits the transport of cholesterol and phytosterols across the brush border membrane of the intestinal enterocyte by blocking the transport function of its molecular target, Niemann-Pick C 1-like 1 protein (NPC1L1), (as reviewed in Ref. 17). Triglyceride and fat-soluble vitamin absorption are unaffected (11). Less cholesterol is transported in chylomicrons, implying a reduction in cholesterol reaching the liver via chylomicron remnants (14–17). Reduced hepatic cholesterol has been reported in ezetimibe-treated mice (14, 18) and dogs (12), and in mice, increases

Abbreviations: apo B, apolipoprotein B; CE, cholesteryl ester; FC, free cholesterol; FCR, fractional catabolic rate; IG, intragastric gavage; LDLR, LDL receptor; NPC1L1, Niemann-Pick C1-like 1 protein; NS, not significant; qRT-PCR, quantitative real-time PCR; TC, total cholesterol; TG, triglyceride.

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in hepatic LDLR expression were observed (18). However, it has not been determined how the molecular mechanisms regulating cholesterol homeostasis in the liver and intestine in response to ezetimibe alter the kinetics of apoB metabolism. Recent apoB-100 kinetic studies demonstrated that in men with primary hypercholesterolemia, ezetimibe decreased LDL-cholesterol 22%, primarily through enhanced fractional catabolic rates (FCRs) of VLDL, intermediate density lipoprotein (IDL), and LDL, suggesting upregulation of hepatic LDLR activity (19).

Inhibition of cholesterol absorption in mice by ezetimibe leads to increases in cholesterol synthesis in intestine and liver (14, 16, 18). Potentially, this would attenuate reductions in hepatic cholesterol, limiting the extent of LDLR expression and ultimately the magnitude of LDL-cholesterol lowering. Thus, combining ezetimibe and a statin would enhance LDL-cholesterol lowering, through complementary mechanisms of action (8, 20). The combination enhances LDL-cholesterol reductions in dogs (12), and in clinical trials, combination therapy decreases LDL-cholesterol to a significantly greater extent than does either agent alone (21, 22). However, the mechanism whereby this combination modulates the kinetics of apoB metabolism has not been investigated. Furthermore, the impact of ezetimibe plus a statin on the expression of genes important for the regulation of LDL metabolism in the liver and intestine has not been defined.

We examined the effect of ezetimibe alone or in combination with simvastatin on apoB-100 kinetics in miniature pigs, a large-animal model of lipoprotein metabolism. Ezetimibe plus simvastatin decreased VLDL and LDL apoB-100 concentrations; ezetimibe inhibited cholesterol absorption, and simvastatin blocked the increase in cholesterol synthesis observed with ezetimibe alone. This resulted in significant reductions of hepatic cholesterol and a synergistic increase in hepatic LDLR expression. Plasma apoB decreased significantly through a modest reduction in VLDL production and a greatly enhanced LDLR-mediated LDL clearance, both of which are attributed to the reduction in hepatic cholesterol.

MATERIALS AND METHODS

Animals and diets

Female pigs (Ja/Mar Farms; Port Dover, Ontario, Canada) weighing 25–30 kg were housed individually in metabolic cages and were fed a pig chow-based diet (600 g/day) containing 34% of calories from fat and 400 mg/day of cholesterol (0.1%, 0.2 mg/kcal). The diet mimics a standard Western-type human diet. The ratio of polyunsaturated to mono-unsaturated to saturated fat (1:1:1) was achieved by mixing lard, butter, and safflower oil (23). Pigs were studied as littermates, three at a time, and matched with respect to baseline total plasma cholesterol. Pigs were randomized to ezetimibe (0.1 mg/kg/day) monotherapy or ezetimibe plus simvastatin (10 mg/kg/day) or placebo (n = 7/group). Blood was obtained through surgically implanted jugular vein catheters in conscious, unrestrained animals (24). The pigs were fasted for 20–22 h prior to blood collection. The protocol was approved by the Animal Care Committee of the University of Western Ontario.

Administration of drugs

Ezetimibe and simvastatin were obtained from Merck-Frosst/Schering Canada, Montreal, Canada. Oral doses of powdered compound were placed in gelatine capsules and fed by hand. In a pilot dose-response study (n = 3 per group), daily doses of ezetimibe at 0.03 and 0.1 mg/kg/day decreased cholesterol absorption by 50% and 76% and reduced LDL-cholesterol by 22% and 30%, respectively, compared with control. In a previous study (24), we demonstrated in this model using the same protocol, that simvastatin at a dose of 10 mg/kg/day decreased LDL-cholesterol by 24%. This dose of simvastatin was chosen because it permitted comparison of the biochemical and kinetic parameters reported previously (24) with those obtained in the present study with ezetimibe alone or in combination with simvastatin. Therefore, ezetimibe (0.1 mg/kg/day) and simvastatin (10 mg/kg/day) were used for the apoB kinetic studies.

Analytical assays

Lipoprotein separations, plasma and lipoprotein lipid analyses, and isolation of apoB have been described previously (23–27). The concentration of apoB in plasma, VLDL, IDL, and LDL was determined by ELISA and confirmed by isopropanol precipitation (25). Lipoprotein cholesterol distributions were evaluated in serum samples (50 μl) from three pigs in each group that were resolved by size exclusion chromatography on a Superose 6 column (28). Plasma lathosterol and plant sterol concentrations were assayed by GC-MS as described previously (29).

Intestinal cholesterol absorption

The plasma dual isotope ratio method described by Turley, Herndon, and Dietschy (30) was modified for use in pigs. The required amounts of [1,2-^{3}H]cholesterol (Amersham; Oakville, Canada) and [4-^{14}C]cholesterol (Amersham) in toluene were evaporated to dryness under nitrogen in separate glass tubes and redissolved in absolute ethanol (1 μl per μCi of radioactivity). The [^{3}H]cholesterol was added dropwise to undiluted 20% Intralipid (0.1 ml per μCi; Baxter Corporation, Toronto, Canada) while vortexing for 3 min. The [^{14}C]cholesterol was added dropwise to safflower oil (0.2 ml per μCi) while vortexing for 3 min. Multiple 10 μl aliquots of each isotope were added directly to 10 ml of Ready Flow III (Beckmann Coulter, Inc.; Fullerton, CA) and counted in a liquid scintillation counter (Beckmann LS3801, Beckmann Coulter, Inc.) to confirm uniform distribution of the tracers in the Intralipid or safflower oil. Following a 22 h fast, pigs were anesthetized using preanesthetics described previously (24) at 9 AM. Each pig received 50 μCi of [^{14}C]cholesterol by intragastric gavage (IG) and 50 μCi of [^{3}H]cholesterol via the indwelling catheter (IV). Immediately upon recovery from the anesthetic (approximately 5 min), animals received their daily dose of placebo, ezetimibe, or ezetimibe plus simvastatin with the standard ration of food. The typical feeding/dosing regimen was continued for 5 days. Plasma samples were obtained every 24 h via the indwelling catheter just prior to feeding. One milliliter of each plasma sample was added directly to the scintillation cocktail and counted to confirm that the plasma decay curves of the two tracers were parallel. The percent cholesterol absorption was calculated from the 72 h plasma samples using the following expression: plasma cholesterol absorption = [percent of IG dose ([^{14}C]cholesterol) per ml plasma] / [percent of IV dose ([^{3}H]cholesterol) per ml plasma] × 100.

Kinetic studies

Pigs received a bolus of trideuterated leucine ([5,5,5-$^{2}H_3$]L-leucine, 3 mg/kg) via the indwelling catheter, following a 22 h

fast (31). Sequential blood samples were obtained, and VLDL, IDL, LDL, and HDL were separated completely by ultracentrifugation. ApoB was obtained by isopropanol precipitation, delipidated, and hydrolyzed to amino acids (29). Leucine was derivatized to the oxazolinone, and isotopic enrichment was determined by GC-MS as described previously (29). Kinetic parameters of apoB-100 metabolism were analyzed using SAAM II (SAAM Institute; Seattle, WA) according to the model reported previously (31).

Tissue analyses

Approximately 22 h after the last dose of drug, pigs were euthanized, and samples of tissues were flash frozen and stored at −80°C. In liver and jejunum, the concentrations of triglyceride (TG), free cholesterol (FC), and cholesteryl ester (CE), and the incorporation of oleate into CE and TG were measured as previously described (27). Hepatic and jejunal microsomal lipids and HMG-CoA reductase activities were determined (27).

Tissue mRNA determination

RNA was isolated using Trizol reagent (Life Technologies; Mississauga, Ontario, Canada). Gene-specific mRNA quantitation for apoB, HMG-CoA reductase, LDLR, SREBP2, SREBP1c, NPC1L1, ABCG5, ABCG8, ABCA1, LXRα, MTP, and GAPDH was performed by quantitative real-time PCR (qRT-PCR) on an ABI Prism (model 7900 HT) Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions (31). Samples (20–75 ng) of reverse transcribed total RNA were assayed in triplicate in 20 μl reactions using a two-step qRT-PCR protocol and the standard curve method to estimate specific mRNA concentrations. Expression levels for each gene were normalized to GAPDH expression levels for each tissue type. For genes with known pig (*Sus scrofa*) sequences (APOB, HMGCR, LDLR, SREBP2, MTP, LXRα, ABCA1, and GAPDH), primer and probe sets were designed using Primer Express 2.0 software (Applied Biosystems) (31). For genes with unknown pig sequences (NPC1L1, ABCG5, ABCG8, and SREBP1c), PCR primers were designed against the mRNA sequence of the human homolog using PrimerQuest software (Integrated DNA technologies). The default settings were used, except that the desired PCR range was set to between 250 and 500 base pairs. PCR primers were tested with pig cDNA prepared using Superscript II reverse transcriptase (Invitrogen) and pig liver or intestine total RNA as per the manufacturer's instructions. PCR products of expected sizes were purified using the QIAquick PCR purification kit (Qiagen), and sequenced on a 3730 DNA analyzer (Applied Biosystems). A minimum of 85% homology of the PCR product sequence to the human sequence was used for verification. Pig-specific primer and probe sets were then designed using Primer Express 2.0. All primers were obtained from Sigma-Genosys, and the fluorogenic probes, labeled with 6-carboxyfluorescein, were obtained from Applied Biosystems. Primers and probes for qRT-PCR analyses are listed in **Table 1**.

Statistical analyses

Differences between control, ezetimibe-, and ezetimibe plus simvastatin-treated animals were compared for statistical significance by paired *t*-test. $P < 0.05$ was considered significant.

RESULTS

Ezetimibe monotherapy reduced plasma total cholesterol (TC) 22% ($P < 0.007$) and LDL-cholesterol 31% ($P < 0.006$) (**Fig. 1**). Ezetimibe plus simvastatin produced significantly greater reductions in both total (−35%, $P < 0.045$) and LDL-cholesterol (−47%, $P < 0.012$), compared with ezetimibe alone (Fig. 1A). Other lipids were unchanged. Ezetimibe decreased VLDL apoB 20% ($P < 0.024$) and LDL apoB 20% ($P < 0.001$) (Fig. 1B). The combination decreased VLDL apoB 26% [not significant (NS) compared with ezetimibe alone], whereas the 34% reduction in LDL apoB ($P < 0.023$) was significantly greater than ezetimibe monotherapy.

Intestinal cholesterol absorption in control pigs was 48%, similar to mean values reported for humans (32). Ezetimibe inhibited absorption 79% ($P < 0.001$) (**Fig. 2A**) and decreased plasma phytosterol concentrations 91% ($P < 0.000001$) (Fig. 2B), demonstrating its efficacy in

TABLE 1. Probes and primer sets used in quantitative real-time PCR in the livers and intestines of control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Probe (5′ to 3′)
APOB	TGAGATCAATCCGCTAGTTCTGAA	CACTGCCGTGCTCTGTTTTC	AATCTGTGAGGTTCTCCA
LDLR	GCTGCATTCCTGAGTCTTGGA	CCGAGTCACAGACAAACTTTGG	CCTGCTGACCGTCACA
HMGCR	TGCTGGTCTGTTTTGATTTTGG	GGATAAGGGCAATTCTTCTCTTGA	ATAGCCAATGTGATTCTT
SREBP2 (SREBF2)	TACGGCTCCTCCATCAATGA	TTGATGTAGTCAATGGCCTTCCT	TTGGCCTCTGTTCCCA
SREBP1c (SREBF1c)	GCCATGGATTGCACGTTC	AAGACAGCGACTTCC	CGTAGGGCGCGTCGAA
ABCA1	TTGTACGAATAGCAGGGTCCAA	GGACGAGGGAAGCTGGTACTG	CCTGACCTGCAGCCG
ABCG5	GGATATTTCTCTGCGGCTCTCTT	GACCACGTTTGGGTCTTGGAT	CACCAAGCAGCACAAG
ABCG8	GCTGGCCAAAGGCAACAG	GGTGGTGCCAGACGTCATC	CTCTTTGACTTGGTCCTC
LXRα(NR1H3)	GGCTGCAGGTGGAGTTCATC	GGCAATGAGCAGGGCAAA	TCGAGTTCTCCAGAGCC
NPC1L1	CGAAGCACAGCGCAACAT	AGTCGGAGAGGCTGGTGTTGT	CAGGACATCTGCTACGC
MTP	GTGGTGGAAGTTCTAGTATGTTGGACTAC	CATATTTTAGAAGTCCAAATGCATCAGTT	TTCAGCCACTCTGGCT
GAPDH	AGGTCGGAGTGAACGGATTTG	ACCATGTAGTGGAGGTCAATGAAG	TCACCAGGGCTGCTT

For genes with known pig (*S. scrofa*) sequences (APOB, HMGCR, LDLR, SREBP2, MTP, LXRα, ABCA1, and GAPDH), primer and probe sets were designed using Primer Express 2.0 software (Applied Biosystems). For genes with unknown pig sequences (NPC1L1, ABCG5, ABCG8, and SREBP1c), PCR primers were designed against the mRNA sequence of the human homologue using PrimerQuest software (Integrated DNA Technologies). PCR primers were tested with pig cDNA prepared using Superscript II reverse transcriptase (Invitrogen) and pig liver or intestine total RNA per the manufacturer's instructions. PCR products of expected sizes were purified using the QIAquick PCR purification kit (Qiagen) and sequenced on a 3730 DNA analyzer (Applied Biosystems). A minimum of 85% homology of the PCR product sequence to the human sequence was used for verification. Pig-specific primer and probe sets were then designed using Primer Express 2.0. All primers were obtained from Sigma-Genosys, and the fluorogenic probes, labeled with 6-carboxyfluorescein, were obtained from Applied Biosystems.

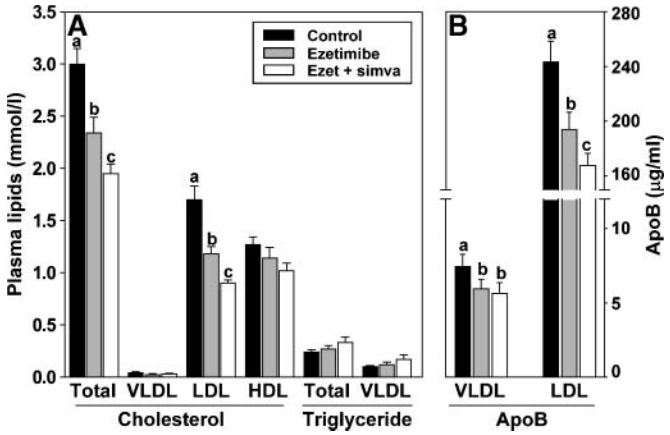

Fig. 1. Plasma lipid, lipoprotein (A), and apolipoprotein (B) concentrations in control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs. Lipid values are the mean ± SEM of three determinations per animal, and apolipoprotein B (apoB) values are the means ± SEM of all samples obtained during the kinetic study from seven animals per group. Bars denoted by different letters are significantly different ($P < 0.05$).

pigs (14, 33). Ezetimibe plus simvastatin had no additional effect on these parameters. Plasma lathosterol levels, a marker of cholesterol synthesis, were increased 65% ($P < 0.044$) by ezetimibe, reflecting the increase in cholesterol synthesis induced by ezetimibe. Lathosterol concentrations decreased to control levels with the addition of simvastatin, reflecting inhibition of hepatic cholesterol synthesis. The ratios of plasma campesterol, sitosterol, and lathosterol to the concentration of plasma cholesterol are shown in Fig. 2C. The ratios showed a pattern of response similar to those of the absolute concentrations of each sterol to each of the treatments.

Trideuterated leucine was injected as a bolus into fasted animals. ApoB kinetic parameters were determined from simultaneous analysis of all isotope enrichment data as described previously (31). A fit of VLDL and LDL apoB enrichment curves for one set of animals is shown in supplementary Figure I (available in the online data supplement). Ezetimibe significantly decreased VLDL apoB pool size 20% ($P < 0.024$); this decrease was attributed to a 27% decrease in VLDL apoB production (NS) (**Table 2**). Ezetimibe plus simvastatin decreased the VLDL apoB pool size 26% ($P < 0.018$), due entirely to a 35% decrease in VLDL apoB production ($P < 0.017$). Neither parameter was significantly different from ezetimibe alone. VLDL FCR, the amount of VLDL apoB converted to LDL apoB, and the amount cleared directly from plasma were not different. IDL apoB kinetic parameters were unchanged (data not shown).

Ezetimibe monotherapy significantly decreased the LDL apoB pool size 20% ($P < 0.001$, Table 2) due to a 42% ($P < 0.013$) increase in the LDL apoB FCR, as total LDL apoB production was unaffected. The combination decreased the LDL apoB pool size 34% ($P < 0.001$) due entirely to an 81% ($P < 0.002$) increase in the LDL apoB FCR. Compared with ezetimibe alone, the combination enhanced the decrease in pool size 70% ($P < 0.003$) and increased the FCR 2-fold ($P < 0.05$). LDL apoB direct synthesis was unaffected. Total apoB production into plasma (VLDL plus LDL direct synthesis) decreased 19% with ezetimibe (NS) and 35% with combination treatment ($P < 0.019$).

VLDL and LDL were analyzed for lipid and protein content (see supplementary Table I). Ezetimibe produced small decreases in the percent of cholesterol in VLDL (NS), whereas the percent of TG increased 4%, ($P < 0.036$). These changes were greater with combination therapy. Ezetimibe decreased the LDL percentage of FC 5% ($P < 0.014$) and increased the percentage of TG and protein 44% ($P < 0.023$) and 11% ($P < 0.022$), respectively. These changes were greater with combination therapy. Other lipid percentages were unaffected. The TG/CE ratio (wt/wt) in LDL increased significantly.

Size exclusion chromatography demonstrated ezetimibe-induced reductions in serum VLDL and LDL-cholesterol, which were enhanced by the addition of simvastatin (**Fig. 3**). Peak elution volumes of cholesterol within each lipoprotein fraction and the ratio of surface to core lipids

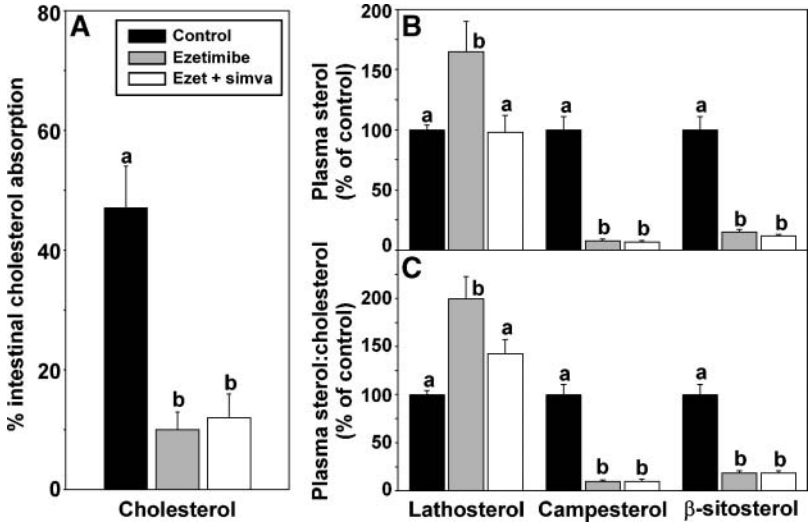

Fig. 2. Fractional cholesterol absorption rates (A), plasma lathosterol, campesterol, and β-sitosterol (B) and the ratios of plasma lathosterol, campesterol, and β-sitosterol to cholesterol (C) in control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs. Control values for lathosterol (2.5 μmol/l), campesterol (73.8 μmol/l), and β-sitosterol (14.0 μmol/l) were set at 100%. Values represent the mean ± SEM from seven animals per group. Bars denoted by different letters are significantly different ($P < 0.05$).

TABLE 2. Metabolic parameters of VLDL and LDL apoB metabolism in control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs

	VLDL Pool Size[a]	VLDL FCR[b]	VLDL Total Production	VLDL Conversion to LDL	VLDL Direct Removal	LDL Pool Size[c]	LDL FCR[d]	LDL Total Production	LDL Direct Production[e]	Total apoB Production[f]
	mg/kg	h^{-1}	*mg/kg/h*	*mg/kg/h*	*mg/kg/h*	*mg/kg*	h^{-1}	*mg/kg/h*	*mg/kg/h*	*mg/kg/h*
Control										
1	0.39	6.85	2.65	0.93	1.45	12.71	0.08	0.99	0.02	2.72
2	0.35	4.55	1.57	1.14	0.42	11.84	0.05	0.59	0.10	1.67
3	0.47	3.73	1.74	0.42	1.30	11.83	0.05	0.59	0.07	1.92
4	0.41	3.67	1.50	0.61	0.88	12.76	0.06	0.78	0.11	1.67
5	0.30	4.49	1.35	1.30	0.04	8.26	0.04	0.33	0.07	1.42
6	0.36	3.79	1.36	0.73	0.62	9.19	0.07	0.64	0.03	1.11
7	0.19	7.04	1.37	1.02	0.34	10.04	0.11	1.06	0.01	1.41
Mean	0.35	4.87	1.65	0.88	0.72	10.95	0.06	0.71	0.06	1.70
SEM	0.03	0.55	0.18	0.12	0.20	0.67	0.01	0.10	0.01	0.20
Ezetimibe (0.1 *mg/kg/day*)										
1	0.25	4.62	1.14	0.73	0.41	9.67	0.08	0.79	0.01	1.20
2	0.17	6.35	1.09	0.45	0.63	10.05	0.06	0.60	0.15	1.96
3	0.45	3.45	1.55	0.95	0.59	10.37	0.09	0.96	0.00	1.55
4	0.33	1.97	0.65	0.63	0.01	9.40	0.07	0.63	0.00	0.65
5	0.27	4.54	1.22	0.48	0.74	7.49	0.09	0.69	0.11	1.43
6	0.32	4.56	1.47	0.69	0.78	7.43	0.10	0.71	0.01	1.50
7	0.18	7.45	1.30	0.98	0.32	6.65	0.15	1.01	0.01	1.33
Mean	0.28	4.70	1.20	0.70	0.50	8.72	0.09	0.77	0.04	1.38
SEM	0.04	0.68	0.11	0.08	0.10	0.56	0.01	0.06	0.02	0.15
P (vs. cont)	0.024	0.759	0.081	0.345	0.399	0.001	0.013	0.504	0.506	0.268
Ezetimibe (0.1 *mg/kg/day*) and simvastatin (10 *mg/kg/day*)										
1	0.18	6.87	1.23	0.49	0.62	6.84	0.15	0.99	0.12	1.35
2	0.16	5.28	0.86	0.49	0.36	8.60	0.10	0.87	0.02	0.89
3	0.44	3.49	1.54	0.73	0.80	8.74	0.14	1.18	0.01	1.56
4	0.35	1.69	0.58	0.56	0.00	7.20	0.08	0.59	0.01	0.61
5	0.21	4.15	0.88	0.57	0.31	6.56	0.12	0.60	0.01	0.91
6	0.32	3.20	1.04	0.65	0.39	6.31	0.11	0.66	0.00	1.05
7	0.16	8.19	1.32	0.77	0.54	6.26	0.13	0.80	0.01	1.35
Mean	0.26	4.70	1.06	0.61	0.43	7.22	0.12	0.84	0.03	1.10
SEM	0.04	0.85	2	0.04	0.10	0.39	0.01	0.08	0.02	0.12
P (vs. cont)	0.018	0.657	0.017	0.102	0.152	0.001	0.002	0.321	0.251	0.019
P (vs. ezet)	0.134	0.987	0.114	0.119	0.591	0.003	0.050	0.313	0.598	0.146

Apo B, apolipoprotein B; cont, control; ezet, ezetimibe; FCR, fractional catabolic rate. Kinetic analysis was performed using SAAM ll.

[a] Pool size refers to the plasma VLDL apoB concentration multiplied by 0.042, (42 ml plasma per kg body weight).

[b] FCR is determined by [Flux(0,6) + Flux (8,6) + Flux (9, 6) + Flux(0,7)]/VLDL apoB pool size, where Flux(0,6) + Flux (8,6) + Flux (9,6) + Flux(0,7) is the production rate of VLDL apoB, and numbers in brackets refer to compartments of the model (see supplementary Figure I).

[c] Pool size refers to the plasma LDL apoB concentration multiplied by 0.042 (42 ml plasma per kg body weight).

[d] FCR is the rate constant k(0,9) determined from the model.

[e] Direct production (direct synthesis) is the production of apoB directly into plasma LDL, compartment 9.

[f] Total apoB production is the production of apoB into the plasma compartment calculated as VLDL apoB production plus LDL apoB direct production.

(see supplementary Table I) were unaffected by either treatment, indicating unchanged lipoprotein sizes.

Ezetimibe decreased liver TC, FC, and CE 19% ($P < 0.018$), 15% ($P < 0.048$), and 36% ($P < 0.022$), respectively (**Fig. 4A**). The combination significantly reduced hepatic TC, FC, and CE (−17%, $P < 0.031$; −12%, $P < 0.046$, and −39%, $P < 0.031$, respectively), but reductions were not different from ezetimibe alone. Compared with control, liver TGs were decreased by 21% ($P < 0.042$) and 19% ($P < 0.047$) by ezetimibe and the combination, respectively. Ezetimibe decreased hepatic microsomal TC (−25%, $P < 0.010$), FC (−21%, $P < 0.050$), and CE (−47%, $P < 0.019$) (Fig. 4B). The combination also reduced hepatic microsomal TC (−29%, $P < 0.003$), FC (−24%, $P < 0.033$), and CE (−65%, $P < 0.009$). Previously, we reported that in pigs treated with simvastatin monotherapy (10 mg/kg/day), liver microsomal FC, CE, and TG were unaffected (24). Ezetimibe decreased oleate incorporation into liver CE (−8%, NS) and TG (−14%, NS). The combination produced significant reductions in oleate incorporation into both liver CE (−18%, $P < 0.013$) and TG (−22%, $P < 0.04$) compared with control (Fig. 4C). In the jejunum, lipid concentrations (see supplementary Figure II) and oleate incorporation into CE and TG (Fig. 4C) were unaffected by either treatment.

Relative mRNA expression of genes involved in hepatic cholesterol metabolism was determined in liver and jejunum. Liver LDLR mRNA increased 1.7-fold ($P < 0.030$) with ezetimibe, and increased further to 3.4-fold ($P < 0.032$ vs. control; $P < 0.048$ vs. ezetimibe) with the combination (**Fig. 5A**). Similar increases were observed in the jejunum (Fig. 5B). Previously, we reported that in pigs treated with simvastatin monotherapy (10 mg/kg/day), liver and jejunum LDLR mRNA both increased 1.3-fold (24). Liver mRNA for HMG-CoA reductase increased 4.1-fold ($P < 0.006$) with ezetimibe, and increased further

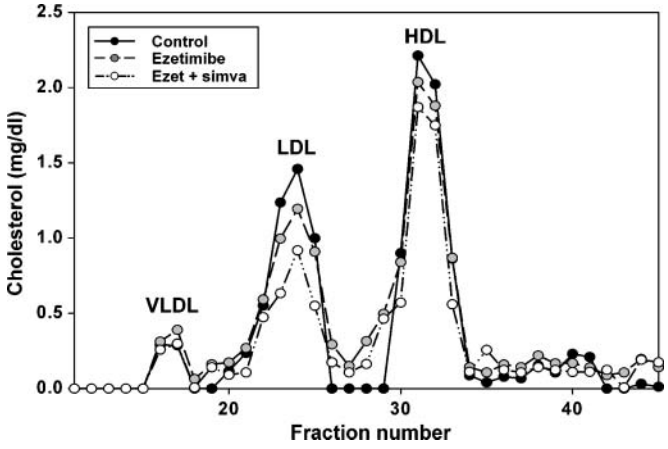

Fig. 3. Lipoprotein cholesterol profiles in serum from control, ezetimibe-, and ezetimibe plus simvastatin-treated pigs. Serum (50 μl) was resolved by size exclusion chromatography using a Superose 6 column. Total cholesterol (TC) concentrations were determined in fraction numbers 11 to 45, with each fraction having a total volume of 500 μl. The plotted values represent the means of three pigs per group, (control, black circles; ezetimibe, shaded circles; ezetimibe plus simvastatin, open circles).

to 13-fold ($P < 0.050$ vs. control) with the combination (Fig. 5A). HMG-CoA reductase activity increased 2.3-fold ($P < 0.002$) in hepatic microsomes of ezetimibe-treated pigs and increased 9.6-fold ($P < 0.006$) in microsomes from the combination group (Fig. 4D). Similar step-wise increases in mRNA and activity of HMG-CoA reductase were observed in the jejunum (Figs. 4D and 5B).

NPC1L1 expression increased in jejunum (1.3-fold, $P < 0.044$) and liver (2.8-fold, $P < 0.049$) with ezetimibe (Fig. 5). The combination increased NPC1L1 expression in jejunum (1.4-fold, NS) and liver (3.1-fold, $P < 0.047$), relative to controls; however, values were not different from ezetimibe alone. Although the expression of NPC1L1 in jejunum relative to liver was 9:1 (data not shown), the enhanced expression of NPC1L1 in liver reflected the degree of tissue cholesterol depletion (Fig. 4).

Ezetimibe increased SREBP2 mRNA in liver 2.0-fold ($P < 0.002$) and in jejunum 1.5-fold. The combination increased SREBP2 mRNA 2.9-fold ($P < 0.006$) in liver and 2.7-fold ($P < 0.034$) in the intestine, compared with control. SREBP1c mRNA was significantly decreased by combination treatment in the liver. All other genes examined, including APOB (Fig. 5), MTP, LXRα, ABCG5, ABCG8, and ABCA1 (see supplementary Figure III) were unaffected.

Figure 6A summarizes the major apoB-100 kinetic parameters observed following ezetimibe and ezetimibe plus simvastatin treatment. VLDL apoB pool size decreased, primarily because of the reduced appearance of newly synthesized VLDL apoB in plasma. LDL apoB concentrations decreased, primarily because of an increased FCR. For comparison, Fig. 6B summarizes the percent change from control (no treatment) in apoB kinetic parameters obtained in the present study compared with parameters determined in a previous study using simvastatin monotherapy (10 mg/kg/day) (24). Although the same animal model and the same protocol were used in both studies, the former study used iodinated tracers, whereas the present study used deuterated leucine to trace apoB. Although this may have resulted in some quantitative differences in calculated parameters, qualitative comparisons of kinetic parameters are possible. Ezetimibe, simvastatin, and the combination all decreased VLDL apoB pool size because of decreased production. Addition of simvastatin to ezetimibe did not appear to enhance these reductions. Ezetimibe alone significantly reduced LDL pool size, owing entirely to an increased FCR. In contrast, simvastatin monotherapy decreased LDL apoB through decreased production, inasmuch as the increases in FCR (+7%) and hepatic LDLR mRNA (+26%) were modest. The addition of simvastatin to ezetimibe enhanced the reduction in LDL apoB pool size, because of a substantial synergistic increase in LDL apoB FCR.

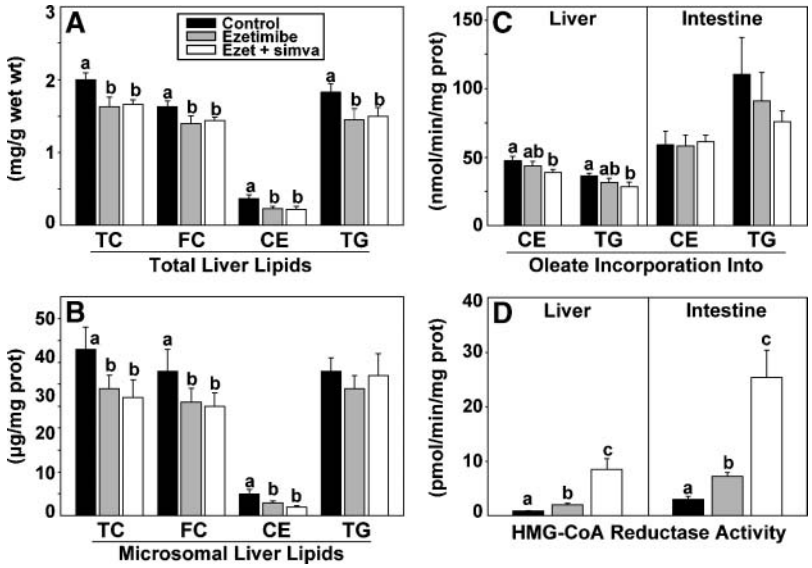

Fig. 4. Tissue lipids, lipid synthesis, and HMG-CoA reductase activities in control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs. TC, free cholesterol (FC), and triglyceride (TG) concentrations were quantitated in whole liver (A) and liver microsomes (B). Oleate incorporation into cholesteryl ester (CE) and TG were determined in homogenates (C). HMG-CoA reductase activity was measured in microsomes (D). Values represent the mean ± SEM from seven animals per group. Bars denoted by different letters are significantly different ($P < 0.05$).

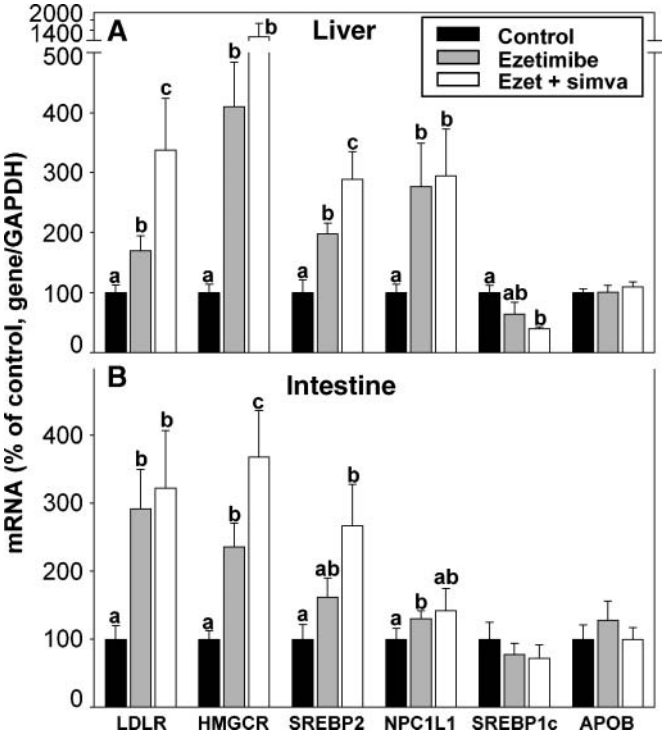

Fig. 5. Tissue mRNA concentrations in control, ezetimibe-, and ezetimibe plus simvastatin-treated miniature pigs. Results are the ratio of the gene of interest relative to GAPDH, and values are expressed relative to control and were set at 100% in liver (A) and in intestine (B). Values represent the mean ± SEM of data from seven animals per group. Bars denoted by different letters are significantly different ($P < 0.05$).

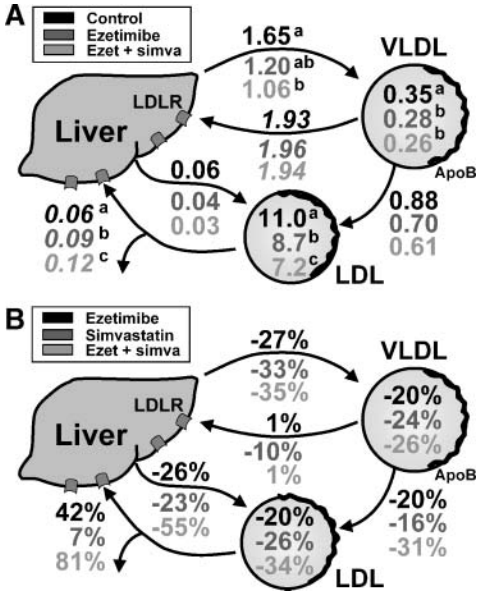

Fig. 6. A: Effects of ezetimibe and ezetimibe plus simvastatin treatment compared with control on apoB metabolism in miniature pigs. Circled numbers represent apoB pool sizes (mg/kg); numbers next to arrows represent mean transport rates (production rates, mg/kg/h), and numbers in italics next to arrows represent fractional catabolic rates (pools/h). Conversion values of apoB to LDL represent apoB from VLDL plus intermediate density lipoprotein. Values denoted by different letters are significantly different ($P < 0.05$). B: Comparison of the percent change from control (no treatment) for the same parameters of apoB metabolism shown in A in pigs treated with ezetimibe, simvastatin alone (24), or ezetimibe plus simvastatin.

DISCUSSION

Inhibition of intestinal cholesterol absorption by ezetimibe has the potential to decrease cholesterol transport to the liver via chylomicron remnants. Reduced hepatic cholesterol would enhance expression of the LDLR, resulting in decreased plasma LDL-cholesterol. However, hepatic cholesterol depletion is accompanied by a compensatory increase in hepatic cholesterol synthesis, making the addition of an HMG-CoA reductase inhibitor an ideal combination for LDL-cholesterol reduction through complementary mechanisms of action. This study demonstrates for the first time in a large-animal model of lipoprotein metabolism the molecular mechanisms underlying the reductions in both VLDL and LDL apoB. The combination of ezetimibe plus simvastatin decreased VLDL and LDL apoB-100 concentrations through reduced VLDL production and enhanced LDLR-mediated LDL clearance. Ezetimibe inhibited cholesterol absorption, and simvastatin blocked the increase in cholesterol synthesis observed with ezetimibe alone, resulting in a significant reduction in hepatic cholesterol and a marked increase in hepatic LDLR expression. Furthermore, we show that expression of NPC1L1 in both intestine and liver is increased with ezetimibe and combination treatment, consistent with regulation by intra-cellular cholesterol.

Previously, we documented reduced VLDL apoB secretion in pigs treated with atorvastatin (24, 25), simvastatin (24), or the oxidosqualene:lanosterol cyclase inhibitor RO-0717625 (31). Reduced VLDL apoB secretion has been linked to decreased availability of cholesterol for hepatic lipoprotein assembly (7, 24, 34). Decreased liver TC and CE in pigs treated with ezetimibe and ezetimibe plus simvastatin is consistent with this concept. VLDL secretion can also be influenced by hepatic TG concentrations, which were reduced by ezetimibe monotherapy. The addition of simvastatin produced no further decrease in liver cholesterol or TG concentrations, consistent with our previous pig studies in which simvastatin monotherapy had no effect on hepatic lipid concentrations (24). The ezetimibe-induced reduction in hepatic TG has also been observed in mice (18) and hamsters (35), and given that ezetimibe does not affect fat absorption (11), decreased hepatic TG is probably secondary to diminished delivery and accumulation of hepatic cholesterol. The contribution of decreased hepatic TG to the diminished VLDL apoB secretion is difficult to determine. Previous studies in this model have demonstrated quantitatively similar reductions in VLDL apoB production, which were correlated with diminished hepatic cholesterol concentrations, yet hepatic TG synthesis and concentrations were unchanged (24, 25, 27, 31). In hypercholesterolemic patients treated with ezetimibe,

Tremblay et al. (19) reported increased VLDL apoB production despite a decrease in VLDL apoB concentrations and increased VLDL apoB FCR. The reason for the increased production is unknown but may reflect the patient type studied or the model used. The efficacy of ezetimibe for LDL-cholesterol reduction in patients with homozygous familial hypercholesterolemia (36), and in LDLR$^{-/-}$ mice (18), also supports a role for ezetimibe in reducing hepatic VLDL production.

Ezetimibe significantly decreased LDL apoB concentrations entirely as a result of enhanced clearance of LDL apoB from plasma, consistent with the significant increase in hepatic LDLR mRNA. This effect was significantly potentiated by simvastatin, producing additive reductions in plasma LDL apoB and synergistic increases in hepatic LDLR mRNA. Enhanced LDL apoB clearance was observed previously in pigs treated with atorvastatin (10 mg/kg/day) (15%) (24), but was unaffected in pigs treated with a lower dose of atorvastatin (3 mg/kg/day) (25) or simvastatin (10 mg/kg/day) (24). In contrast, inhibition of the apical sodium-dependent bile acid transporter decreased LDL apoB as a result of significant increases in LDL apoB FCR, 18% in monotherapy and 45% in combination with atorvastatin (27, 37), demonstrating that combining drugs with complementary mechanisms of action can amplify LDL apoB clearance.

Our finding that ezetimibe monotherapy decreases liver cholesterol has been reported in ezetimibe-treated wild-type and LDLR$^{-/-}$ mice fed a lipid-rich diet (18). Consistent with the present study, these authors reported a 2-fold increase in hepatic LDLR mRNA in wild-type mice; however, LDL apoB kinetics were not reported. In ezetimibe-treated LDLR$^{-/-}$ mice, it was concluded that ezetimibe decreased LDL-cholesterol primarily through reduced VLDL apoB production, although the latter was not measured directly (18). Our results with ezetimibe monotherapy are consistent with those of Tremblay et al. (19), where decreased LDL-cholesterol in patients with primary hypercholesterolemia treated with ezetimibe was due to enhanced LDL apoB clearance. We now demonstrate that this is due to increased hepatic LDLR expression consequent to diminished hepatic cholesterol. Furthermore, addition of simvastatin enhanced the effect of ezetimibe monotherapy by further increasing LDLR mRNA and LDL apoB clearance 2-fold, thus providing the molecular mechanism for the enhanced decrease in LDL-cholesterol observed in patients treated with ezetimibe plus a statin.

As anticipated, ezetimibe increased hepatic and intestinal HMG-CoA reductase expression and activity (14, 18), consistent with the increased plasma lathosterol levels, a marker of cholesterol synthesis. Increased plasma lathosterol has been reported in patients treated with ezetimibe (13). Elevated hepatic HMG-CoA reductase expression and cholesterol synthesis has been observed in ezetimibe-treated mice (16). This compensatory increase in cholesterol synthesis probably attenuates the increase in LDLR expression and reductions in plasma LDL-cholesterol. We observed that addition of simvastatin greatly increased the expression and activity of HMG-CoA reductase in liver and intestine. This anticipated effect of statins is the result of a positive feedback regulatory mechanism, mediated by enhanced activity of the transcription factor SREBP-2. The increased HMG-CoA reductase activity reflects statin removal during microsomal preparations (24). Enhanced ex vivo HMG-CoA reductase expression and activity have been reported previously in pigs treated with simvastatin or atorvastatin, and the extent of increase in ex vivo reductase activity was proportional to the extent of cholesterol synthesis inhibition in vivo (24). In the present study, addition of simvastatin was sufficient to inhibit the ezetimibe-induced increase in cholesterol synthesis as plasma lathosterol concentrations decreased to control levels with combination therapy.

A wide range of inter-individual variability in the LDL-cholesterol response to ezetimibe has been observed (38), and this almost certainly encompasses attenuation of the LDL-cholesterol-lowering effect of ezetimibe over time in some patients. Although the molecular mechanisms are unclear, it is possible that individuals who might experience ezetimibe tachyphylaxis have relatively greater compensatory upregulation of hepatic cholesterol synthesis than do patients whose LDL-cholesterol response remains stable with time. Patients who show attenuation of the LDL-cholesterol response to ezetimibe may thus be ideal candidates for combination therapy with statins, although more investigation would be required to substantiate this concept.

LDLR and HMG-CoA reductase expression is primarily regulated by SREBP2. SREBP2 mRNA increased significantly in both the liver and intestine of pigs treated with ezetimibe and increased even further with the combination. This provides a mechanism for the increased expression of both of these sterol-responsive genes. The expression of SREBP1c was decreased in liver, and although this provides a potential explanation for the decrease in hepatic TGs with both treatments, the mechanism is unclear, perhaps reflecting diminished hepatic cholesterol levels.

NPC1L1 expression was increased in both the jejunum and liver by ezetimibe and was increased further by the combination. These increases reflected cholesterol depletion in both tissues, consistent with transcriptional regulation by sterols via sterol-regulated elements within the NPC1L1 promoter (16). This suggests that its expression is regulated similarly to that of other genes coding for proteins involved in cholesterol metabolism, including LDLR and HMG-CoA reductase (39). This upregulation may have increased the tissue content of NPC1L1 transporters; however, there appeared to be sufficient ezetimibe for inhibition of NPC1L1-mediated sterol absorption, as evidenced by the approximately 80% reduction in cholesterol absorption and 91% decrease in plasma phytosterols following treatment. In contrast to mice and rats, human liver also expresses NPC1L1 (16, 40). Although expression of NPC1L1 in pig intestine is 9-fold higher than in liver, increased expression was observed in both tissues. The physiological significance of NPC1L1 in hepatocytes remains to be elucidated. The apical location of NPC1L1 in hepatoma cells predicts a canalicular distribution of NPC1L1 in vivo,

which has been confirmed in monkey liver (41). Both enterocytes and hepatocytes are polarized cells with their apical surface exposed to micelles containing cholesterol. NPC1L1 mediates intestinal cholesterol absorption from micelles in the intestinal lumen, and it is tempting to speculate that it may also promote cholesterol reuptake from micelles in the canalicular bile. Furthermore, in species that express hepatic NPC1L1, including humans and pigs, the pharmacological efficacy of ezetimibe may be partially attributed to the blocking of this proposed canalicular reuptake mechanism (41).

In conclusion, inhibition of NPC1L1 by ezetimibe and inhibition of cholesterol synthesis by simvastatin in the pig results in significant reductions in LDL apoB-100 concentrations relative to monotherapy. Ezetimibe inhibited cholesterol absorption and simvastatin blocked the increase in cholesterol synthesis observed with ezetimibe alone, resulting in a significant reduction in hepatic cholesterol and a marked synergistic increase in hepatic LDLR expression. Plasma apoB was significantly decreased by a modest reduction in VLDL apoB production and a greatly enhanced LDLR-mediated LDL apoB clearance, both of which are attributed to the reduction in hepatic cholesterol. Therefore, inhibition of NPC1L1 by ezetimibe enhances LDL lowering compared with statins alone, through a distinct, yet complementary mechanism of action. These studies support the findings of recent clinical trials in which enhanced reductions of LDL-cholesterol achieved by combining ezetimibe with statin therapy greatly facilitated the ability of patients to reach new, more-stringent LDL-cholesterol targets.

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