Enhancement of Fatty Acid and Cholesterol Synthesis Accompanied by Enhanced Biliary But Not Very–Low-Density Lipoprotein Lipid Secretion Following Sustained Pravastatin Blockade of Hydroxymethyl Glutaryl Coenzyme A Reductase in Rat Liver

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A 3-week treatment of rats with pravastatin (PV) augmented biliary cholesterol and phospholipid output 3.6- and 2.2-fold over controls, while bile acid (BA) output and kinetics were unchanged. No major changes were detected in hepatic and serum cholesterol concentrations despite the PV inhibitory property on hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase. To evaluate the mechanisms of this adaptive phenomenon, several parameters of hepatic lipid homeostasis were assessed. Biliary cholesterol changes could not be attributed to an increased influx of lipoprotein cholesterol to the liver and bile. Hepatic low-density lipoprotein (LDL) receptor content, as inferred from Western blot analysis, was unchanged, as was the biliary excretion of labeled cholesterol derived from chylomicron remnants. In vivo ^3H_2O-incorporation studies showed an 80% increase in hepatic cholesterol synthesis, evidence for bypass of the PV block. Remarkably, fatty acid synthesis was also stimulated twofold, providing substrate for hepatic triglycerides, which were slightly enhanced. However, serum triglycerides decreased 52% associated with a 22% decrease in hepatic very–low-density lipoprotein (VLDL) secretion. Thus, the biochemical adaptation following PV treatment produces complex alterations in hepatic lipid metabolism. An enhanced supply of newly synthesized cholesterol and fatty acids in association with a limited VLDL secretion rate augments the biliary lipid secretion pathway in this experimental model.

CONSISTENT AMOUNTS of lipids, mostly represented by cholesterol and phospholipid, are continuously transferred from the hepatocyte interior to the canalicular membrane from which they are solubilized by bile acids (BAs) and secreted into bile.[1,2] Several hepatic metabolic processes have been found to be linked to biliary cholesterol and phospholipid secretion,[2,3] but this relationship is incompletely characterized. Aside from the rates of very–low-density lipoprotein (VLDL) secretion and cholesterol esterification,[4-6] probably acting as modulators of biliary lipid secretion, a major role in the supply of biliary lipids is played by hepatic cholesterol synthesis and especially the uptake of plasma lipoproteins. In rat studies using radiolabeled precursors, newly synthesized cholesterol has been demonstrated as an active source of biliary cholesterol and a preferential substrate for BA formation.[3,7,8] In contrast, no change in biliary cholesterol and BA output could be detected under several physiological manipulations to inhibit or stimulate the rate of hepatic cholesterol synthesis.[8,9]

More recently, the relationship between hepatic cholesterol synthesis and biliary secretion has been studied using a group of compounds named statins,[10] acting as competitive inhibitors of hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase, the limiting enzyme in the cholesterol synthetic pathway. Kempen et al[11] and Pandak et al[12] showed that administration of a single large intravenous (IV) bolus of statins such as lovastatin or mevinolin to rats previously subjected to prolonged biliary drainage markedly decreased the biliary output of both cholesterol and BAs. The physiological relevance of these studies, though, is tempered by the known side effects of sustained biliary drainage on intestinal cholesterol absorption and the activity of key enzymes of hepatic cholesterol metabolism.[13] In another study, Bilhartz et al[14] observed no changes in biliary lipid secretion in rats fed a mevinolin diet for 1 week, but as food was withdrawn, they noted a parallel increase in both the biliary lipid secretion rate and hepatic cholesterol synthesis. Although interpreted as a "rebound" effect related to a decreased mevinolin concentration at the regulatory sites upon cessation of feeding, the findings suggested the induction of more extensive metabolic changes by this class of medications. That there was a true rebound-independent increase of biliary lipid secretion with statin treatment has been clearly shown in subsequent studies using suitable experimental conditions.[15,16] In addition, evidence has been obtained for the occurrence of metabolic changes along with the increased biliary lipid secretion, such as increased hepatic cholesterol synthesis,[14,17-20] suggesting the occurrence of more extensive adaptive effects consequent to treatment with statins.

In the present study, the major parameters of hepatic cholesterol homeostasis were studied in relation to biliary lipid and BA secretion. A rat model was used that is characterized by a short-term bile-fistula following prolonged and uninterrupted administration of pravastatin (PV), a hydrophilic statin acting on the liver. Our findings demonstrate a complex resetting of hepatic lipid homeostasis in response to PV administration, which ultimately led to increased biliary lipid secretion. To this end, several mechanisms were identified besides stimulated cholesterogenesis, among which are an increased fatty acid synthesis and a decreased VLDL secretion rate without changes in hepatic lipoprotein uptake.

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MATERIALS AND METHODS

Animals and Treatment

Male Sprague-Dawley rats weighing 220 to 280 g were used after adaptation to a 12-hour light-dark cycle for 2 weeks. The experimental treatment consisted of a 3-week ad libitum dietary administration of 0.075% (wt/wt) PV[21] thoroughly mixed in a standard ground chow (MIL mice and rats Standard Diet; Stefano Morini, San Polo D'Enza, Italy) containing 0.064% (wt/wt) cholesterol. For each experiment, a PV-treated group and ground chow–fed control group were studied in parallel. Unless otherwise noted, all biliary and hepatic studies were started at the mid-dark phase of the light cycle, ie, at a time when rats usually consume most of their food and hepatic cholesterol synthesis and biliary lipid secretion reach their highest rates. Food withdrawal was not applied prior to any procedure except as otherwise indicated. At the end of the experiments, animals were killed by diethyl ether inhalation or IV pentobarbital overdose. The study protocol was approved by the University Committee for Animal Research. PV was kindly provided by Bristol Myers-Squibb (Princeton, NJ). Body and liver weight and daily food intake (~20 g/rat) were comparable between treated and control groups (Table 1).

Biliary Secretion Experiments

For biliary secretion experiments, animals were lightly anesthetized by diethyl ether inhalation and a biliary fistula was surgically implanted as previously described.[22] A PE-10 catheter was also inserted in a tail vein for continuous infusion of 0.15 mol/L NaCl at a rate of 1.0 mL/h and lipoprotein injections. Timed bile collections were started immediately after surgery and continued for up to 24 hours, during which time the animals were kept in restraining cages and received only drinking water, no food. For the assay of total BAs, a sample of bile was extracted in spectrophotometric-grade methanol (J T. Baker, Deventer, The Netherlands) for 10 minutes at 37°C, and a 3αOH-steroid dehydrogenase–based method was applied to the clear supernatant after sample centrifugation at low speed for 5 minutes at 4°C.[23] Phospholipids were measured as an inorganic phosphorus–ammonium molybdate complex after digestion in aqueous 70% perchloric acid at 180°C for 90 minutes[24] and are reported as lecithin. Biliary (free) cholesterol was determined following lipid extraction[25] by a cholesterol oxidase method (Boehringer, Mannheim, Germany; kit no. 310328).[26]

Table 1. Effects of PV on Body Weight, Liver Weight, Liver Lipids, and Serum Lipids

Parameter	PV-Treated	Control
Body weight (g)	208 ± 6	205 ± 4
Liver weight (g)	7.95 ± 0.36	8.77 ± 0.47
Relative liver weight (% body weight)	3.82 ± 0.11	4.27 ± 0.17
Liver cholesterol		
Total		
mg/g	3.10 ± 0.10*	2.68 ± 0.09
mg/organ	24.99 ± 1.58	23.88 ± 1.38
Free		
mg/g	2.54 ± 0.08	2.36 ± 0.04
mg/organ	20.28 ± 1.24	20.48 ± 0.97
Esters		
mg/g	0.56 ± 0.04*	0.38 ± 0.07
mg/organ	4.71 ± 0.45	3.40 ± 0.79
Liver triglycerides		
mg/g	5.01 ± 0.62	3.99 ± 0.31
mg/organ	39.05 ± 4.41	35.88 ± 5.96
Serum cholesterol (mg/dL)		
Total	95.80 ± 7.69	90.63 ± 3.84
Free	21.85 ± 1.44	22.59 ± 1.19
Esters	73.60 ± 6.68	68.04 ± 2.83
Serum triglycerides (mg/dL)	26 64 ± 2.22*	51 33 ± 7.02

NOTE. Upon completion of the experimental feeding period, groups of PV-treated rats (n = 9) and control rats (n = 12) were anesthetized by diethyl ether without any prior fasting, and the blood and liver were obtained for assay of lipids. Results are the mean ± SEM.

*$P < .05$.

BA Kinetics

A number of parameters describing BA metabolic kinetics were derived from the pattern of BA excretion into bile (the washout method)[27] by applying the following formulas: (1) BA secretion rate (micromoles per 24 hours per 100 g of body weight) derived from the BA secretion rate obtained in the first 90 minutes of biliary drainage; (2) BA synthesis (micromoles per hour per 100 g of body weight) considered equal to the lowest point of BA secretion after drainage of the endogenous BA pool for about 9 to 12 hours; (3) BA pool (micromoles per 100 g body weight) representing the cumulated mass of BA collected from the start of biliary drainage until the time point immediately preceding that corresponding to the BA synthetic rate; and (4) BA cycling (number per 24 hours) calculated by dividing the value for the BA secretion rate into the value for the BA pool.

Assay of Serum and Liver Lipids

At the end of the feeding period, the animals were subjected to diethyl ether anesthesia, blood was collected by aortic puncture, and the liver was removed and exhaustively rinsed in ice-cold 0.15-mol/L NaCl/1.0-mmol/L EDTA, pH 7.4. The blood was centrifuged at low speed at 4°C to separate the serum. For the purpose of VLDL preparation, serum was combined with 0.1% (wt/vol) Thimerosal and centrifuged in an SW-41 Beckman (Fullerton, CA) rotor for 18 hours at 274,000× *g* and 15°C. The top 1.0 mL (VLDL fraction) was quantitatively recovered by a tube cutter. Serum and lipoprotein free cholesterol was assayed by the same enzymatic procedure as for bile,[26] while triglycerides and total (free plus esterified) cholesterol were determined by other enzymatic commercial kits (Boehringer no. 887557 and 290319). To determine liver lipids, a 25% (wt/vol) liver homogenate was prepared in ice-cold 0.15-mol/L NaCl/1.0-mmol/L EDTA, pH 7.4, using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Lipids were extracted in chloroform/methanol 2:1 (vol/vol),[25] and triglyceride and free cholesterol were assayed by the above-described enzymatic kits. For the measurement of cholesteryl esters, an additional aliquot of lipid extract was hydrolyzed for 90 minutes in 0.8 mol/L ethanolic KOH, and cholesterol was extracted in hexane and quantified enzymatically as before.[26] The cholesteryl ester concentration was derived by the difference in free cholesterol between the hydrolyzed and unhydrolyzed lipid extract.

Immunodetection of Hepatic LDL Receptor Protein

At the completion of experimental feeding, treated and control animals (n = 10 per group) were killed under ether anesthesia and the liver was obtained. A liver lobe was dissected out, rinsed with ice-cold NaCl 0.15 mol/L, and homogenized by Polytron in 4 vol ice-cold buffer consisting of 10 mmol/L Tris, 0.15 mol/L NaCl, 1.0 mmol/L $CaCl_2$, 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF), pH 7.4. A crude liver membrane preparation was obtained by differential centrifugation exactly as previously described.[28] All procedures were performed at 4°C. The membrane pellet was resuspended in 0.25 mol/L Tris maleate, 2.0 mmol/L $CaCl_2$, and 1.0 mmol/L PMSF, pH 6.0, sonicated twice for 15 seconds, incubated for 10 minutes in the same buffer containing 30 mmol/L CHAPS and 50 mmol/L NaCl, and cleared by a 60-minute centrifugation at 100,000 × *g*. Solubilized proteins were subjected to sodium dodecyl sulfate gel electrophoresis and electrotransferred onto

nitrocellulose membrane. LDL receptor protein was reacted with a specific polyclonal antibody (generously provided by Dr Allen C. Cooper, Palo Alto Medical Foundation, Palo Alto, CA) and subsequently immunostained with peroxidase-conjugated goat anti–rabbit immunoglobulin G antibodies.[28] After washing, the color was developed by the Immuno-Blot (GAR-HRO) assay kit (BioRad, Richmond, CA) and quantified by densitometric scanning (Hoefer Scientific Instruments, San Francisco, CA).

Biliary Excretion of Chylomicron Remnant–Derived Cholesterol

In these studies, a trace of ^{3}H-cholesterol–labeled chylomicron remnants was administered as an IV bolus immediately upon completion of bile-fistula surgery, and the time course of label incorporation in biliary neutral (cholesterol) and acidic (BA) sterols was evaluated for up to 24 hours. Labeled chylomicrons were obtained from rat lymph under a duodenal infusion of lipid emulsion containing [1,2-^{3}H(N)]-cholesterol, and their remnants were prepared in vivo as previously described.[29] Labeled lipoproteins were dissolved in 0.15-mol/L NaCl/1.0-mmol/L EDTA, pH 7.4, with added penicillin 1×10^6 U/mL and gentamycin 0.3 mg/mL, and kept at 4°C and under N^2 for up to 10 days from preparation. Before injection, lipoproteins were dialyzed against four changes of 0.15-mol/L NaCl 3L for a period of 12 hours. The radioactive counts administered as ^{3}H-remnants were 2.4×10^6 dpm/100 g body weight. Upon extraction by chloroform/methanol 2:1 (vol/vol)[25] and lipid separation by thin-layer chromatography (TLC) on silica gel G (Merck, Bracco, Milan, Italy) under development in hexane/diethyl ether/glacial acetic acid 86:16:1 (vol/vol), ^{3}H-remnant radioactivity was distributed 70% in esterified and 30% in free cholesterol. The biliary secretion of the injected lipoprotein radioactivity and its partition into biliary neutral and acidic sterols were studied according to a described procedure.[30] For neutral sterols, a volume of bile was placed into a glass-stoppered conical tube and extracted twice in methanol/hexane 3:20 (vol/vol). Aliquots of pooled hexane extracts were subjected to radioactive counting after addition of Instagel (Packard, Downers Grove, IL) in a liquid scintillation spectrometer equipped with external standardization. To evaluate radioactivity in acidic sterols, the aqueous layer left over the neutral sterol extraction was alkalinized with 8 mol/L KOH and heated for 3 hours at 125°C in tightly stoppered thick glass vials to deconjugate BAs. After cooling and acidification to pH 3 with 6 mol/L HCl, bile acids were extracted twice in diethyl ether 10 vol. The pooled extracts were dried, redissolved in 1.0 mL methanol, and counted similarly as before after color quenching with 30% H_2O_2.

^3H_2O Incorporation Into Hepatic Cholesterol and Fatty Acids

To this end, treated and control rats were injected IV with 10 mCi ^3H_2O brought to 0.15 mol/L NaCl in a total volume of 1.0 mL, and a published procedure was used.[31] Sixty minutes later and under diethyl ether anesthesia, the aortic blood and liver were obtained. The blood was placed in heparinized tubes and centrifuged at low speed at 4°C for 20 minutes to obtain the plasma, an aliquot of which was counted in Instagel (Packard) to determine the plasma water specific activity. The livers were first flushed via the portal vein with 20 mL ice-cold 0.15-mol/L NaCl/1.0-mmol/L EDTA, pH 7.4, and then excised, blotted on absorbent paper, minced, and homogenized in 4 vol of the same buffer with four passes of a teflon pestle driven at full speed in a Potter Hevelhjem homogenizer (Glas-Col, Terre Haute, IN). Quadruplicate portions of homogenate, each corresponding to 0.4 g liver, were extracted in chloroform/methanol 2:1 (vol/vol)[25] and hydrolyzed in 0.875 mol/L ethanolic KOH for 1.5 hours at 80°C. To determine ^3H_2O incorporation in cholesterol, the ethanol concentration was halved by the addition of water, and nonsaponifiable lipids were extracted by shaking for 2 minutes with three subsequent 15-mL portions of petroleum ether (boiling point, 40° to 60°C). To prepare digitonides, compounded extracts were dried under N_2, redissolved in 5.0 mL acetone/ethanol 1:1 (vol/vol), acidified with 1 drop of 1.0 mol/L HCl, and treated with 2.0 mL 1.0% (wt/vol) digitonin in 50% ethanol. Following overnight standing, the digitonin precipitate was collected by a 15-minute low-speed centrifugation, and washed twice with acetone and once with diethyl ether. After 10 minutes at room temperature and then 1 hour at 70°C under a fume hood, the precipitate was dissolved in methanol and an aliquot was counted in 10 vol Picofluor (Packard). To account for a possible digitonin coprecipitation of sterols other than cholesterol, ^3H_2O incorporation in cholesterol was also determined by direct high-performance liquid chromatography (HPLC) isolation[32] and counting of this moiety. To measure ^3H_2O incorporation in fatty acids, the aqueous residue left over the previous nonsaponifiable lipid extraction was acidified with H_2SO_4 and fatty acids were extracted with two subsequent portions of 15 mL petroleum ether (bp 40° to 60°C). Extracts were pooled, dried under N_2, and redissolved in hexane, and an aliquot was placed in scintillation vials, dried, and counted in Picofluor. To verify the specificity of label incorporation into fatty acids, aliquots of final extracts were subjected to TLC on silica gel G developed in hexane/diethyl ether/glacial acetic acid 90:22.5:2.5 (vol/vol). The fatty acid band was localized by the mean of external standards, visualized by I_2 vapor, and scraped from the plate. Lipids were eluted from the silica with chloroform, transferred to scintillation vials, dried, and counted in Picofluor.

VLDL Triglyceride Secretion Rate and Apparent Turnover

An established method was used in these experiments.[33] Rats were fasted for 12 hours to eliminate any plasma inflow of chylomicron triglyceride, and then injected IV with Triton WR1339 (600 mg/kg in 1.0 mL 0.15 mol/L NaCl) to inhibit plasma VLDL catabolism. A 0.3-mL blood sample was drawn from a tail vein into heparinized capillary tubes before and 90 minutes after the injection, the plasma was separated, and triglyceride levels were measured by an enzymatic commercial kit. The following parameters were determined: (1) triglyceride secretion rate (TGSR) in milligrams per minute = $Tg(90) - Tg(0) \times Plv$ and (2) fractional catabolic rate (FCR) per minute = $TGSR/Tg(0) \times Plv$, where Tg(0) and Tg(90) are plasma triglycerides (milligrams per milliliter) before and 90 minutes after Triton WR1339 injection and Plv is the plasma volume (taken as 4% of body weight).

Data Analysis

All experimental data are reported as the mean ± SEM. Statistical evaluation of differences between mean values was performed by an unpaired Student's *t* test.

RESULTS

Serum and Hepatic Lipids

Table 1 compares serum and liver lipid levels in control and treated animals. As reported by others[34] and also consistent with the effects found for biliary lipid secretion, serum and liver cholesterol levels were not decreased by treatment, indicating that adaptive changes had occurred to compensate for the inhibition of HMG CoA reductase. On a per-gram-liver basis, total and esterified liver cholesterol were increased 15.7% and 47% over controls ($P < .05$), but these differences decreased to 4.6% and 38.5% and became unsignificant when lipid levels were related to liver weight. Liver free cholesterol was unaltered. Serum triglycerides decreased 51.9% under treatment (26.64 ± 2.22 *v* 51.33 ± 7.02 mg/dL, $P < .05$), at variance with liver triglycerides, which were slightly elevated, although

unsignificantly. Triglycerides also decreased 62% in the serum VLDL fraction (data not shown).

Biliary Lipid Secretion

PV administration resulted in drastic changes in biliary lipid secretion that were apparent from the onset of biliary drainage, thus representing a true rebound-unrelated effect. During the initial 3 hours, lipid secretion (micromoles per 100 g body weight) in PV-treated versus control animals was 1.959 ± 0.249 versus 0.540 ± 0.021 for cholesterol ($P < .005$) and 9.525 ± 0.303 versus 4.365 ± 0.381 for phospholipid ($P < .05$). Secretion changes in cholesterol and phospholipid were substantially maintained over the entire 24-hour experimental period (Fig 1). In addition, the fold-increases in cholesterol and phospholipid secretion varied, respectively, from 3.6- and 2.2-fold in the initial 3 hours to a minimum of 2.1-fold and twofold at 9 hours, and increased again thereafter with a peak of 3.2- and 5.2-fold at 15 hours. It is suggested that this late secretory peak may relate to or identify with the "rebound" increase in biliary lipids and cholesterol synthesis described by Bilhartz et al[14] during 6 to 12 hours of biliary drainage in an experiment of programmed withdrawal of a lovastatin-containing diet. However, it is to be noted that the cited study[14] included additional experimental manipulations such as an initial unaccounted drainage of biliary components and glycocholate infusion, which render it not readily comparable to the present study. At variance with biliary cholesterol and phospholipid, BA output was minimally increased in the initial 3 hours (21%, P = NS), even though it tended to increase with time (Fig 1), becoming twofold of control levels at 18 hours, possibly reflecting a bile-fistula–related derepression of BA synthesis.[13,35] The biliary lipid concentration showed a trend for changes similar to that for secretion (Table 1), even though treatment-related differences appeared less evident. In the initial 3-hour period, treated animals showed a 35% increase in bile flow (0.501 ± 0.016 *v* 0.370 ± 0.012 mL/h/100 g body weight, $P < .001$), a change that persisted throughout the experiment (Table 2). Variations in BA kinetics, of potential consequence on the biliary lipid secretion rate, were evaluated by the washout method.[27] Although BA synthesis appeared slightly increased in relation to treatment (0.786 ± 0.064 *v* 0.501 ± 0.110 μmol/h/100 g body weight, $P < .05$), there were no changes in the BA pool size (41.72 ± 3.27 *v* 34.14 ± 4.23 μmol/100 g body weight, P = NS) or recycling (5.13 ± 0.36 *v* 5.12 ± 1.00 cycles/24 h, P = NS). To further examine the role of BAs in the lipid secretion process, cholesterol and phospholipid output during the initial 12 hours were each plotted against BA output and found to best fit a rectangular hyperbolic equation, as also observed in other studies.[4] The maximal biliary lipid secretion rates obtained by

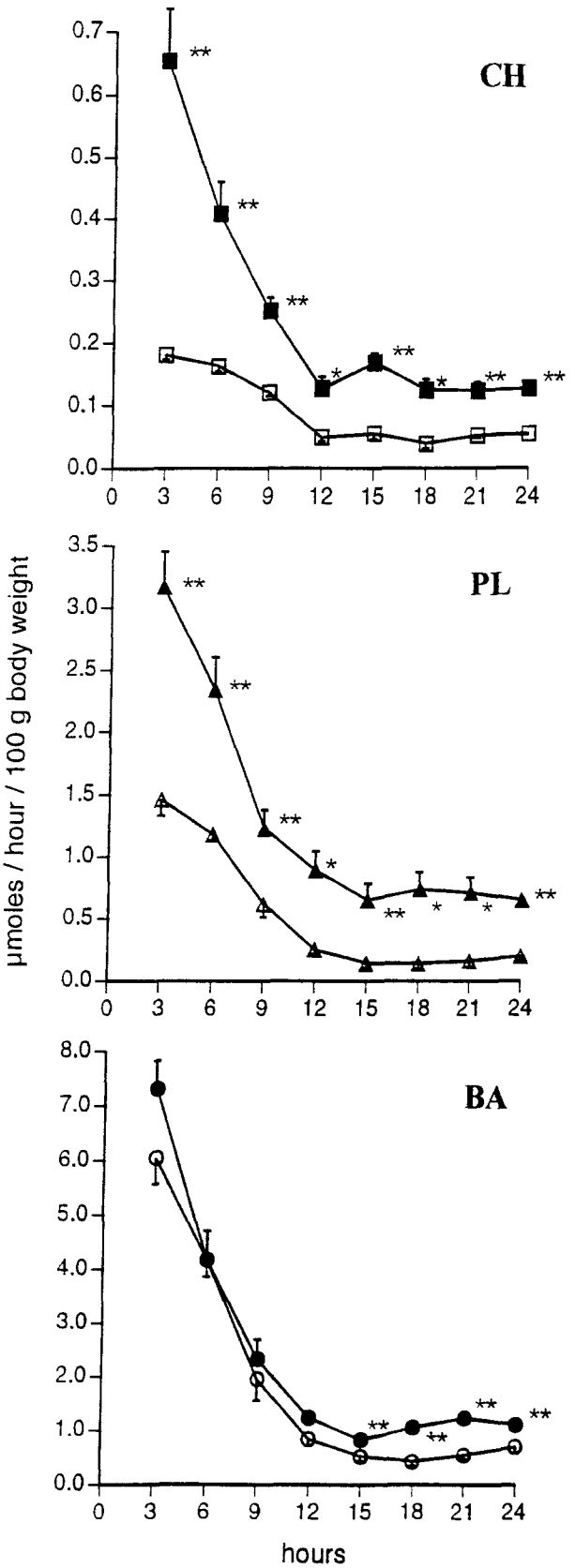

Fig 1. Time course of the biliary output of cholesterol (CH), phospholipid (PL), and BA in rats treated with PV and in controls. Male Sprague-Dawley rats treated for 3 weeks with 0.075% (wt/wt) PV mixed in regular ground chow diet (closed symbols, n = 8) and a group of matched controls (open symbols, n = 6) underwent a bile-fistula procedure at the middle-dark phase of the light cycle without any prior fasting. Timed bile collections were started immediately after surgery. The biliary output of CH, PL, and total BA is reported as μmol/h/100 g body weight (mean ± SEM). Statistical significance by Student's *t* test: *$P < .05$ and **$P < .005$.

Table 2. Time Course of Biliary BA, Cholesterol, and Phospholipid Concentrations and of Bile Flow in Relation to PV Treatment

Parameter		Collection Interval (h) 0-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24
BA									
PV	Mean	6.449	4.110	2.815	1.712	1.273	1.543	1.817	1.629
	SEM	0.304	0.450	0.355	0.192	0.070	0.098	0.071	0.110
Control	Mean	6.684	5.515*	3.184	1.686	1.203	0.961*	1.201*	1.593
	SEM	0.728	0.293	0.574	0.220	0.189	0.131	0.207	0.294
Cholesterol									
PV	Mean	0.580	0.408	0.304	0.230	0.187	0.181	0.181	0.180
	SEM	0.079	0.053	0.031	0.022	0.023	0.019	0.009	0.011
Control	Mean	0.215†	0.214*	0.198*	0.110†	0.112†	0.089*	0.115†	0.124*
	SEM	0.005	0.002	0.006	0.018	0.010	0.015	0.010	0.021
Phospholipid									
PV	Mean	2.812	2.352	1.486	1.244	0.963	1.084	1.026	0.925
	SEM	0.261	0.289	0.172	0.243	0.186	0.174	0.111	0.058
Control	Mean	1.725*	1.573*	1.007	0.495*	0.338*	0.333*	0.359*	0.459†
	SEM	0.126	0.087	0.147	0.062	0.069	0.067	0.114	0.110
Bile flow									
PV	Mean	0.501	0.442	0.372	0.320	0.297	0.302	0.288	0.296
	SEM	0.016	0.021	0.015	0.012	0.015	0.018	0.019	0.025
Control	Mean	0.370†	0.322†	0.257†	0.212†	0.185†	0.182†	0.195†	0.190†
	SEM	0.012	0.019	0.011	0.010	0.005	0.006	0.008	0.010

NOTE. Experimental setup and data are the same as in Fig 1. BA, cholesterol, and phospholipid concentrations are expressed as μmol/mL/100 g body weight; bile flow is mL/h/100 g body weight.

*$P < .05$.

†$P < .005$.

this method were significantly increased by the treatment from 0.25 ± 0.03 to 1.22 ± 0.33 ($P < .05$) for cholesterol and from 2.74 ± 0.40 to 5.35 ± 1.32 ($P < .05$) for phospholipid. Thus, in treated animals, increased amounts of cholesterol and phospholipid were secreted into the bile per unit of BA (Fig 2).

LDL Receptor Protein and Remnant-Derived Cholesterol Elimination Into Bile

In theory, both biliary and hepatic lipid changes could reflect an enhanced hepatic input of lipoprotein lipids. The present study has specifically examined this previously unaddressed aspect. First, the expression of hepatic LDL receptor involved in the binding of apolipoprotein (apo) B/E-containing lipoproteins[36-38] was studied. However, LDL receptor protein abundance showed only a marginal increase, which did not reach statistical significance (3,342 ± 357 *v* 2,718 ± 336 densitometric units in treated *v* control animals). In keeping with these data are the unmodified VLDL triglyceride clearance rate or LDL receptor protein abundance shown under lovastatin[20] or atorvastatin[39] feeding. Secondly, lipoprotein cholesterol transport into bile was examined following IV injection of ^{3}H-cholesterol–labeled chylomicron remnants. Label excretion was then determined in the neutral and acidic biliary sterol fractions. Chylomicron remnants were chosen in view of their active hepatocyte uptake and catabolism, mediated by both LDL[36] and LRP[37-38] receptors.[34-36] There were no differences in the biliary appearance and recovery of ^{3}H-labeled neutral sterols. The 24-hour excretion of biliary neutral sterols (percentage of administered radioactivity) reached 3.244 ± 0.315 and 3.219 ± 0.402 in control and treated animals. However, the radioactivity recovered in the acidic sterol fraction was significantly reduced by treatment, an effect lasting throughout the experiment that led to a 58% ($P < .005$) decrease in the 24-hour output of biliary acidic sterols (from 7.471 ± 0.684 in controls to 3.354 ± 0.511 in treated animals, % of administered radioactivity). Taken together, the findings on the LDL receptor level and biliary secretion of remnant-derived cholesterol appear to exclude an increase in hepatic lipoprotein cholesterol inflow as a determinant of the increased biliary cholesterol output (Fig 3).

Hepatic Cholesterol and Fatty Acid Synthesis

Assessment of hepatic lipid synthesis provided complementary findings to the biliary lipid changes (Table 3). Specifically, label incorporation into hepatic cholesterol was increased 80% over controls as measured by digitonide precipitation or to 145% when assessed by HPLC, a finding extending previous observations.[17-20] Furthermore, a PV-related increase of similar magnitude (~twofold) was also demonstrated in the hepatic fatty acid synthetic rate, whether measured in the total lipid extract or TLC-separated fatty acids. Changes in cholesterol and fatty acid synthetic rates remained essentially unvaried when incorporation data were referred to liver weight. By this calculation, the incorporation rates into cholesterol digitonides and total fatty acid extracts (micromoles per hour per liver) were 42.35 ± 4.90 versus 25.84 ± 2.09 (+63.8%, $P < .05$) and 75.57 ± 9.34 versus 39.93 ± 3.89 (+89%, $P < .01$) in PV-treated and control groups. These findings clearly establish the development of multiple hepatic adaptive biochemical changes following chronic PV intake, involving not only cholesterol synthesis through a presumable bypass of the drugs' block on HMG CoA reductase but also other hepatic lipid synthetic processes, such as fatty acid synthesis.

VLDL Secretion Rate

To assess further the significance of plasma triglyceride changes induced by PV, VLDL triglyceride secretion was

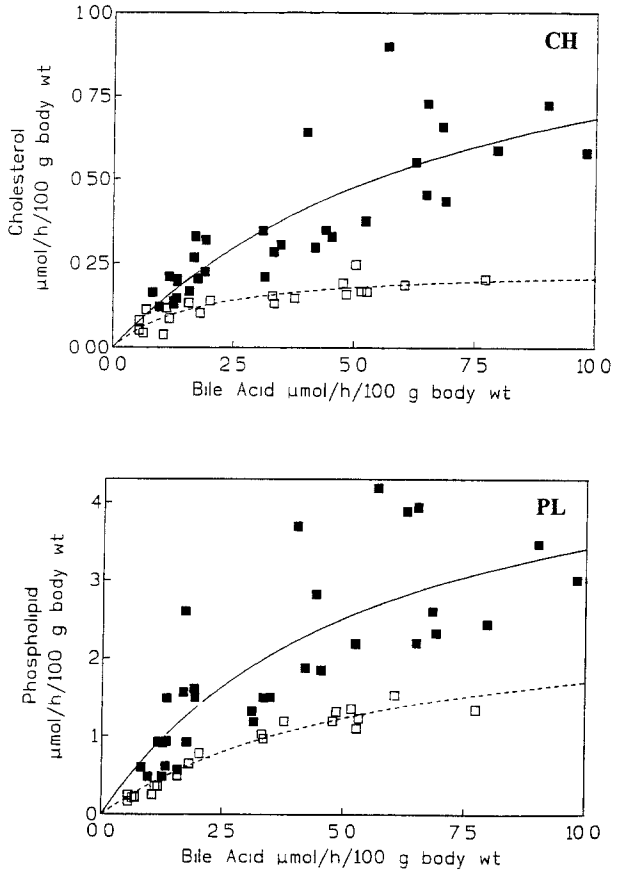

Fig 2. Kinetic relationships between biliary lipid and BA secretion. The biliary output of cholesterol (CH) and phospholipid (PL) during the initial 12 hours of bile-fistula was plotted against BA output in both control (□) and PV-treated (■) rats. Experimental data, the same as in Fig 1, were best fitted to a rectangular hyperbolic equation, y = ax/b + x, where a is the maximal lipid secretion rate and b represents BA secretion at the half-maximal lipid secretion rate. Equations for CH and PL secretion, respectively, were y = (0.25 ± 0.03)x/(1.85 ± 0.58) + x and y = (2.74 ± 0.40) x/(6.13 ± 1.52) + x in controls, and y = (1.22 ± 0.33)x/(7.81 ± 3.55) + x and y = (5.35 ± 1.32)x/(5.66 ± 2.60) + x in treated animals. The term a was significantly different ($P < .05$) between control and treated animals for both CH and PL secretion.

evaluated. The TGSR (milligrams per minute) was significantly decreased by PV treatment from 1.205 ± 0.062 to 0.940 ± 0.025 ($P < .05$), while the FCR (per minute) appeared unchanged (0.266 ± 0.011 *v* 0.299 ± 0.008 in treated and control animals, P = NS). These data indicate a decrease in the VLDL secretion rate induced by the administered drug, and are in contrast to the increased availability of hepatic lipids suggested by the increase in both the lipid synthetic rates and hepatic cholesteryl ester and triglyceride levels (Table 4).

DISCUSSION

The first finding and starting point of this study was the increase associated with PV treatment in the biliary secretion of cholesterol and phospholipid (3.6- and 2.2-fold over control levels, respectively), evident since the outset of biliary drainage. No change was shown in either BA secretion or the enterohepatic pool size and cycling to explain these findings. Furthermore, when the secretion rates of biliary lipids were plotted against those of BA, more cholesterol and phospholipid appeared to be secreted into the bile per mole of BA. According to current concepts, the increased lipid supply to the canalicular membrane determined by PV administration could derive from an increase in hepatic lipoprotein uptake and/or lipid synthesis;

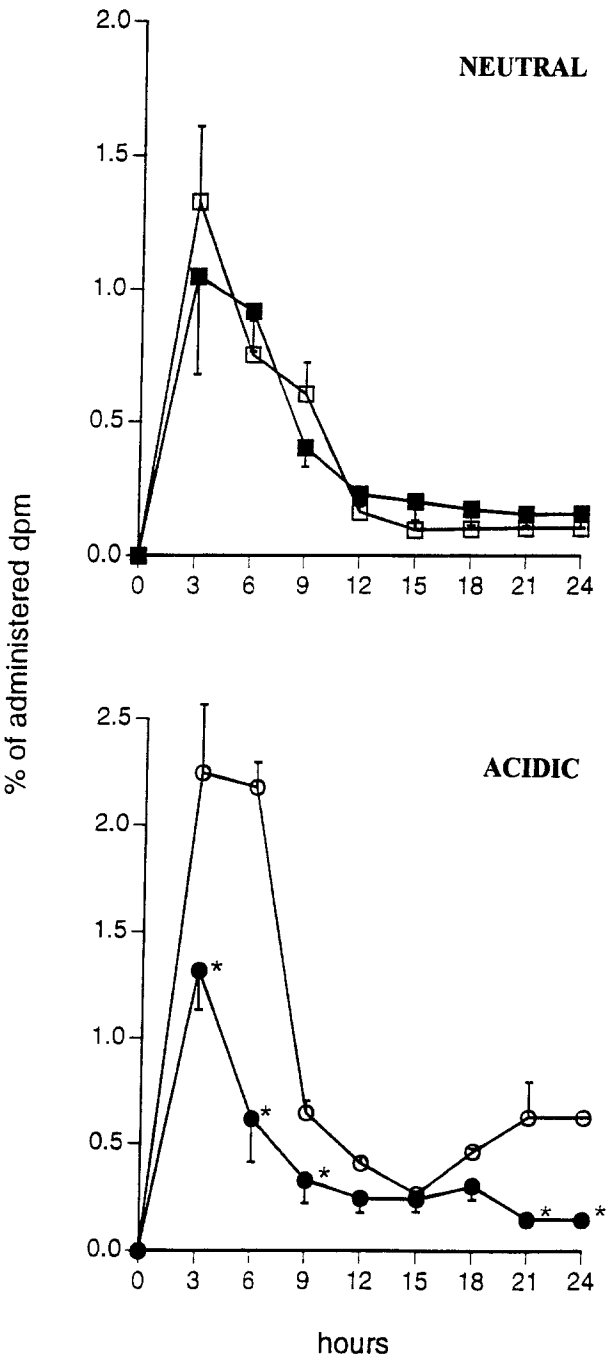

Fig 3. Time course of the biliary excretion of chylomicron remnant-derived ^{3}H-cholesterol in association with neutral and acidic sterols. PV-treated rats (closed symbols, n = 5) and control rats (open symbols, n = 4) underwent a bile-fistula procedure in the fed state and under diethyl ether anesthesia. A trace of ^{3}H-cholesterol-labeled chylomicron remnants was injected by IV bolus upon completion of surgery, and timed bile collections were readily started. An IV infusion of 0.15 mol/L NaCl was maintained throughout the experiment, but no feeding was allowed. Labeled neutral sterols and acidic sterols were extracted from bile fractions and counted. Data are the mean ± SEM. *$P < .05$.

Table 3. Effects of PV on ^3H_2O Incorporation Into Hepatic Cholesterol and Fatty Acids

Group		Cholesterol		Fatty Acids	
		Digitonides	HPLC	Total Extract	TLC
PV	Mean	5,327*	1,396*	9,506*	6,513*
	SEM	621	223	1,175	1,120
Control	Mean	2,946	570	4,553	3,414
	SEM	241	174	444	377

NOTE. PV-treated rats (n = 7) and control rats (n = 6) were injected IV with ^3H_2O 10 mCi. One hour later, a sample of blood was taken from the aorta and the liver was flushed with cold 0.15 mol/L NaCl and excised. ^3H_2O incorporation in cholesterol was measured by both digitonin precipitation and HPLC; the incorporation in fatty acids was determined on both the total extract and after TLC separation. Incorporation rates are reported as nmol/g/h.

*$P < .05$.

on the other hand, a change in the other major pathway of lipid export from the hepatocyte, the VLDL secretion rate, could be involved.[4,5] The present study was able to examine these and other potential determinants of the biliary lipid secretion changes occurring in relation to PV treatment.

First, no significant variation was found in the abundance of the hepatic LDL receptor, a membrane protein involved in the hepatic uptake and catabolism of apoB/E lipoproteins including LDL and the intestinally derived chylomicron remnants. This finding is at variance with human studies in which both LDL receptor binding and protein are increased by statins,[40-42] a response that appears mostly responsible for the decreased serum LDL levels consequent to this treatment. On the other hand, the absence of a coordinated downregulation of the hepatic LDL receptor protein in the presence of an increased hepatocyte cholesterol level and synthetic rate is an intriguing finding also described in other studies,[43] thought to represent a metabolic regulation specific to rat liver. In accordance with the data on LDL receptor protein levels, biliary excretion of chylomicron remnant–derived ^{3}H-cholesterol was unaffected by the treatment. It is acknowledged that exchange of labeled cholesterol with other hepatic cholesterol pool(s) might prevent accurate precursor-product measurement in the current experiments. Overall, data on both the hepatic LDL receptor protein and biliary cholesterol excretion strongly suggest that there was no increase in the hepatic uptake of lipoprotein lipids to account for the observed changes in biliary lipid output. In the ^{3}H-cholesterol–labeled remnant studies, PV treatment was also accompanied by a decreased recovery of labeled BA, a finding that could represent either an impaired conversion of remnant cholesterol to BA or a disturbed BA hepatocanalicular transport. While the first possibility appears inconsistent with the previously discussed BA biliary secretion rate and metabolic kinetics, the second may relate to PV competition with BA hepatocyte uptake at the level of the Na^+-independent multispecific organic anions transporter.[44] However, resolution of these points requires further investigation.

Table 4. PV-Related Changes in the Secretion Rate and Apparent Turnover of VLDL Triglyceride

Group		Triglyceride (mg/mL)		TGSR	
		0 min	90 min	(mg/min)	FCR/min
PV	Mean	0.327*	8.180	0.940*	0.266
	SEM	0.015	0.330	0.025	0.011
Control	Mean	0.452	9.719	1.205	0.299
	SEM	0.024	0.384	0.062	0.008

NOTE. Following a 12-hour food withdrawal, treated and control rats (n = 7 per group) were injected IV with Triton WR1339 to inhibit plasma VLDL degradation. Plasma triglyceride accumulation over 90 minutes was taken as a measure of hepatic VLDL triglyceride secretion (TGSR). The triglyceride FCR was derived secondarily.

*$P < .05$.

Of most importance are the experiments that examined the synthetic sources of biliary lipids. Hepatic cholesterol synthesis was enhanced 80% in livers from treated animals compared with controls, implying that HMG CoA reductase blockade by the drug was bypassed. On the other hand, the significance of the statin-related increase of hepatic cholesterogenesis as a true adaptive phenomenon was clearly apparent in the early studies by Khan et al,[20] and has been confirmed in several more recent observations.[17-19] It has been shown that the reduced formation of sterols or nonsterol mevalonate derivatives subsequent to the blockade of mevalonate production by statins is followed by a release of HMG CoA reductase protein from its synthetic and degradative controls, resulting in a massive accumulation of this enzyme.[21] These data collectively suggest that although statins are inhibitory in acute experiments,[10,34,45] their chronic administration induces an adaptive increase in the mass of HMG CoA reductase up to a level at which the enzyme would start to respond again to stimulatory factors, ie, fatty acids.[46] A compensatory increase in hepatic HMG CoA reductase and in vivo cholesterol synthesis along with an increased biliary cholesterol output have also been shown in the hamster in relation to mevinolin feeding,[47,48] with an associated increase in bile lithogenicity and frequency of gallstone formation.[47] In contrast, normal men and gallstone patients undergoing long-term mevinolin[49] or PV[40,50] treatment show a decrease in biliary cholesterol secretion or lithogenicity, possibly reflecting a lower level of hepatic cholesterol synthesis as compared with rats both physiologically[3] as well as under statin administration.[40,49] However, an additional major finding of the present study is the twofold stimulation of hepatic fatty acid synthesis accompanying that of cholesterol under prolonged PV administration, consistent with studies in which lovastatin has been found to increase acetyl CoA carboxylase, the enzyme catalyzing malonyl CoA formation in the first committed step of fatty acid synthesis.[51] The significance of the increased fatty acid synthesis in the context of the present study is manyfold: (1) It suggests a mechanism by which the PV inhibitory block of HMG CoA reductase may be overcome through an increased formation of intermediate precursors, among which are acetyl CoA, acetoacetyl CoA, and especially HMG CoA; and (2) The enhanced availability of fatty acid substrate may also be the basis for an increased synthesis of phospholipid to be eventually translocated to the exo-leaflet of the canalicular membrane[52] and then released into the bile. Indeed, increased phosphatidylcholine synthesis has been shown during rat feeding with mevinolin[17] or PV.[53]

VLDL production is known to parallel the increased hepatic availability of fatty acids such as oleate or cholesterol,[54] and to decrease during fasting, where mitochondrial fatty acid oxida-

tion occurs. In the present study, which was also performed in the fed state, there was an increased hepatic flux of newly synthesized fatty acid and cholesterol, leading to an increase in cholesteryl esters and triglycerides. Notwithstanding the availability of both cholesterol and fatty acid substrates, VLDL secretion was decreased by PV treatment, an effect also noted in previous studies in rats[20,34,55,56] and humans[57] under treatment with different statins. However, availability of lipid substrates is only one among multiple factors and mechanisms determining the VLDL secretion rate.[58] The reduced VLDL secretion rate at the sinusoidal pole of the hepatocyte and the simultaneous increase in neosynthetic hepatic lipid inflow deserve further consideration regarding their role in the activation of the biliary lipid secretion pathway.

In conclusion, prolonged PV administration in the rat was shown to induce a number of hepatic adaptive responses centered on an increased synthesis of both cholesterol and fatty acid. The increased hepatic inflow of newly synthesized lipids, coupled to an unsuppressed lipoprotein uptake and decreased VLDL secretion, provide the conditions for an increased lipid flow to bile. Together, these observations delineate a model to understand the biochemical regulations interrelating hepatocyte lipid synthesis and VLDL secretion with the mechanisms coordinating cholesterol and phospholipid transport to bile.

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