CHAPTER 5

3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE INHIBITORS INDUCE HEPATIC EXPRESSION OF THE PHOSPHOLIPID TRANSLOCASE Mdr2 P-glycoprotein in Rats

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ABSTRACT

Biliary cholesterol secretion is coupled to that of phospholipids in a process controlled by \textit{Mdr2} P-glycoprotein activity and bile salt secretion. Statins, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have been shown to affect hepatobiliary lipid secretion in rats. The aim of this study was to relate effects of statins on bile formation to the expression of \textit{Mdr2} and other hepatic adenosine triphosphate-dependent transport proteins involved in bile formation in rats. Rats received simvastatin- or pravastatin-containing chow continuously for 5 days. In one group of rats, simvastatin was withdrawn at 9-12 hours prior to the end of experiments to induce biliary cholesterol hypersecretion (rebound). Bile and liver tissue were collected for lipid analysis, and hepatic messenger RNA and protein levels were studied by reverse transcriptase-polymerase chain reaction, immunoblotting and immunohistochemistry. Simvastatin-feeding did not alter biliary bile salt secretion. The secretion of phospholipids and cholesterol was stimulated by 74% and 90%, respectively, in the simvastatin-continuous group and by 72% and 235%, respectively, in the rebound group compared with controls. \textit{Mdr2} mRNA levels increased only in the continuous group. \textit{Mdr2} protein levels were increased in both simvastatin-fed groups. Induction was most pronounced in periportal hepatocytes. \textit{Mdr1b} mRNA levels were moderately increased in both simvastatin-fed groups. Levels of other hepatic transport proteins did not change. Similar results were obtained in pravastatin-fed rats. We conclude that statins increase expression of \textit{Mdr2} and \textit{Mdr1b} in rats, revealing a novel effect of these commonly used drugs.
INTRODUCTION

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis. These drugs are widely used in the treatment of hypercholesterolemic patients. Statins lower plasma LDL cholesterol in humans by: (i) enhanced clearance of LDL by transcriptional upregulation of hepatic LDL receptors, and/or (ii) reduced production of LDL by either decreasing its secretion into the plasma or by decreasing the conversion of VLDL to LDL. Statins do not lower plasma cholesterol in rodents that carry most of their plasma cholesterol in the HDL fraction. Yet, statins have been shown to affect hepatobiliary lipid secretion in rats. It was demonstrated that long-term (>5 days) statin-treatment results in induction of hepatic HMG-CoA reductase expression and enzyme levels, and promotes biliary lipid output. In addition, cessation of statin-treatment leads to a marked hypersecretion of cholesterol into bile within 6-12 hours, accompanied by a relatively smaller increase in biliary phospholipid secretion. Bile salt secretion, on the other hand, is not influenced. As both cholesterol and phospholipid secretion are coupled to that of bile salts, the statin-induced secretion of lipids indicates that these drugs exert specific actions in the liver. The hypersecretion of cholesterol into bile after statin withdrawal has been attributed to an inappropriately high hepatic cholesterol synthesis, due to the induction of the HMG-CoA reductase system. Evidently, this does not explain the concomitant hypersecretion of phospholipids.

Recently, it has become clear that phospholipid secretion into bile is controlled by mdr2 P-glycoprotein (Pgp) in the bile canalicular membrane. Mdr2-Pgp is a member of the Pgp-subfamily of ATP-binding cassette (ABC) transport proteins, which in rodents comprises at least four members that are all localized in the canalicular domain of hepatocytes: Mdr1a, Mdr1b, Mdr2, and Bsep. The expression of Mdr2 in normal rodent liver is high and evidence indicates that this protein functions as a flippase that translocates phosphatidylcholine across the membrane. In contrast to Mdr2, Mdr1a and Mdr1b are present at low levels in normal rat liver. Overexpression in cells of the latter two proteins confers multidrug resistance against a broad variety of natural product drugs. The physiological function of Mdr1a and Mdr1b in the liver presumably involves hepatobiliary transport of bulky amphiphilic compounds, including hydrophobic peptides, steroids, and, perhaps, of certain lipids. Bsep, the product of the so-called sister gene of Pgp, is abundantly present in rat canalicular membranes and has recently been shown to function as the major bile salt export pump. The Multidrug Resistance Protein (MRP1) and its isoforms are members of another subfamily (MRP/CFTR) of the ABC protein superfamily. The human MRP1, and its rat homologue Mrp1, are expressed at low levels in normal liver. The recently cloned Mrp1 homologue Mrp2 has a similar substrate specificity as Mrp1 and is highly expressed in the canalicular membrane domain of hepatocytes and functions as cmoat (canalicular multispecific organic anion transporter). Mrp2 has been implicated in the removal of pravastatin from the body by mediating its secretion into bile.

To gain insight in the mechanism(s) underlying statin-associated changes in biliary lipid secretion, we have evaluated the effects of continuous and discontinuous administration of simvastatin on the expression of genes encoding hepatic ABC transport proteins. For comparison, rats were continuously exposed to pravastatin or to diosgenin, another established inducer of cholesterol hypersecretion.
EXPERIMENTAL PROCEDURES

ANIMALS
Pathogen-free male Wistar rats (270-310 g) were purchased from Harlan, Zeist, the Netherlands. They were housed at the Central Animal Facility of the University of Groningen in a temperature-controlled environment with alternating 12-h light (6:00 am to 6:00 pm) and dark cycles. All experiments were performed between 7:00 am and 9:00 am. The rats received standard laboratory chow (RMH-B; Hope Farms BV, Woerden, The Netherlands) and had free access to food and water. The study was approved by the Local Committee for Care and Use of Laboratory Animals.

EXPERIMENTAL DESIGN
Three independent animal experiments were performed. **Experiment A:** Rats were fed for five days either the standard control diet (control, \(n=10\)) or the same diet supplemented with 0.1% (w/w) simvastatin (Merck, Sharp & Dome Inc., Whitehouse Station, NJ). At the end of the five-day period one of the simvastatin-supplemented groups was returned to the control diet 9-11 hours before the end of the experiment (simvastatin-rebound; SR group, \(n=10\)) while the other group was maintained on simvastatin-supplemented diet (simvastatin-continuous; SC group, \(n=10\)). In the morning of the sixth day, rats were anaesthetized with pentobarbital (60 mg/kg, intraperitoneally). Of each group, three animals were subjected to bile duct catheterization to allow collection of bile. After a 5 min stabilization period, bile was collected for 30 min and stored at -20 °C until further analysis. The livers were perfused with phosphate buffered saline (PBS), excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen and stored at -80 °C until used for isolation of membranes or of RNA. For immunohistochemistry, small pieces of liver were frozen in isopentane and stored at -80 °C until further use. Blood samples were collected in EDTA-containing tubes and centrifuged immediately (10,000 g) and plasma was stored at -20 °C until used. **Experiment B:** The above mentioned experiment was repeated as described, except that simvastatin was replaced by pravastatin (Bristol-Myers Squibb, Princeton, NJ), and that a ‘rebound’ group was omitted. Rats were fed for five days either the control diet (control, \(n=3\)) or the same diet supplemented with 0.1% (w/w) pravastatin (pravastatin-continuous; PC group, \(n=5\)). At the end of the five-day period bile was sampled, rats were killed and tissue was collected as described for the rats in the experiment with simvastatin. **Experiment C:** Rats were fed for 5 days with either the standard control diet (control, \(n=3\)) or the same diet supplemented with 0.5% (w/w) diosgenin, a plant saponin (Sigma Chemical Co. (St. Louis, MO)) (diosgenin, \(n=3\)). At the end of the five-day period bile was sampled, rats were killed and tissue was collected as described for the rats in the experiment with simvastatin.

ANTIBODIES
A mouse monoclonal antibody C219 (Signet Laboratories Inc, Dedham, MA) was used for detection of all P-glycoproteins (Pgps). This antibody recognizes a conserved epitope close to the ATP-binding cassette in all known members of the Pgp-subfamily. Polyclonal antibodies against Mrdr (k111) were raised by immunoizing rabbits with the peptide EEEVEELSDK coupled to Keyhole limpet hemocyanin (KLH) via the NH2-terminus, and was used for the detection of Mrdr on immunoblots. Mouse monoclonal antibody P3-II-26 was kindly provided by Drs G.L. Scheffer and R.J. Scheper (Free University, Amsterdam, The Netherlands). This antibody has been described in preliminary form and was used for the immunohistochemical detection of rat Mrdr P-glycoprotein. On immunoblot k111 and P3-II-26 recognize a 170 kD protein in canalicular membrane-enriched fractions from rat liver, but not in basolateral membrane-enriched fractions. In addition, a clear, single band was observed in membrane subfractions from insect cells infected with a recombinant baculovirus expressing the rat Mrdr gene, whereas no specific signals were detected in membranes from insect cells overexpressing the rat Mrdr1b, human MDR1, or human MRP1 genes (data not shown). Polyclonal antibodies against Bsep (k12) and Mrp2 (k4) were raised in our laboratory.
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MRNA ANALYSIS BY RT-PCR
Total RNA was isolated from frozen rat liver using TRIzol Reagent (GIBCO BRL, Grand Island, NY) according to manufacturer's instructions. Subsequently, mRNA was isolated using the Oligotex mRNA mini-kit (Qiagen GmbH, Hilden, Germany). Single stranded cDNA was synthesized from 2.5 µg mRNA and subsequently subjected to relative polymerase chain reactions as described by our laboratory. Specific primer sets for the various hepatic ABC proteins were designed and for each primer set an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal number of cycles to be used. This was determined to be halfway through the exponential phase. For every PCR reaction, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as internal control. The number of cycles were 22 for Gapdh and Mrp2, 24 for Bsep, 29 for Mdr1b and Mdr2, and 33 for Mdr1a and Mrp1. Primer sequences and product sizes are previously described. Sequence analysis confirmed the specificity of the PCR-primers for each PCR-product. In each experiment, water was used as a negative control. Ten microliters of PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using a CCD video camera of the ImageMaster VDS system (Pharmacia).

ISOLATION OF MEMBRANES FROM LIVER HOMOGENATES
Crude liver cell membrane fractions and canalicular membrane-enriched fractions were isolated by sucrose gradient centrifugation techniques according to Meier and Boyer, with some modifications as detailed by Vos et al. For later use, membrane fractions were stored at -80 °C in 10 mM Tris/HCl, pH 7.4 with Complete protease inhibitor cocktail (1:50 weight/volume, Boehringer Mannheim GmbH, Mannheim, Germany) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma).

IMMUNOBLOT ANALYSIS
The crude cell membrane proteins (100 µg) or canalicular membrane-enriched proteins (15 µg) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Amersham International, Buckinghamshire, UK), using a tankblotting system according to manufacturer’s instructions (BioRad Laboratories, Hercules, California). BDH molecular weight standards (42,700-200,000 Dalton range, BDH Ltd, Dorset, UK) were used as marker proteins. The blots were stained with Ponceau S-solution (0.1% Ponceau S (w/v) in 5% acetic acid (v/v), Sigma) to confirm similar protein concentrations in every lane. The blots were incubated with the first antibody diluted in PBS containing 4% non-fat dried milkpowder (Fluka BioChemica, Buchs, Switzerland) and 0.05% polyoxyethylene sorbitan monolaurate (Tween-20, Sigma), washed in PBS / 0.05% Tween 20, subsequently incubated with horse-radish peroxidase labeled swine anti-rabbit IgG or rabbit anti-mouse IgG (dilution 1:2000, DAKO A/S, Glostrup, Denmark) and finally developed using Pierce SuperSignal Chemiluminescent Substrate Luminol/Enhancer (Pierce, Rockford, IL).

IMMUNOHISTOLOGY
For immunohistochemical studies, 4 µm-sections were cut from frozen liver tissue. After drying, sections were fixed in acetone for 10 min at room temperature. The monoclonal antibodies C219 and P3II-26 and the polyclonal antibody k12 were diluted (1:50, 1:250 and 1:50, respectively) in PBS containing 1% bovine serum albumin (BSA). Sections were incubated with 50 ml of the diluted antibody for 60 min. After incubation with the first antibody, endogenous peroxidase activity was blocked by incubating the sections in 50 ml PBS containing 125 ml of a 30% H2O2 solution for 30 min at room temperature. Slides incubated with the monoclonal antibodies C219 and P3II-26 were subsequently incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (dilution 1:50) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:50) for another 30 min. Slides incubated with the polyclonal antibody k12 were subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution 1:100) for 30 min followed by an incubation with peroxidase-conjugated
rabbit anti-goat IgG (dilution 1:100) for another 30 min. All peroxidase conjugated immunoglobulins were diluted in PBS containing 1% BSA and 5% normal rat serum. Peroxidase activity was developed with 3-amino-9-ethylcarbazole solution (0.2 mg/ml sodium acetate buffer containing 0.03% H₂O₂) for 15 min at room temperature. Nuclear counter-staining was performed with hematoxylin and the slides were covered with Kaiser’s glycerin-gelatin. All antibody incubations were performed at room temperature, and after each incubation, sections were rinsed with PBS.

ANALYTICAL PROCEDURES

Protein concentrations were determined with the DC Protein Assay (BioRad Laboratories) using BSA as standard. Bile salt concentrations were determined by an enzymatic fluorometric assay (Sterognost-Flu, Nyegaard & Co., Oslo, Norway). Triglycerides, cholesterol, cholesterylester and phospholipids were measured in liver homogenates or bile after lipid extraction according to Bligh and Dyer as described by Kuipers et al. Plasma cholesterol and triglycerides were measured using a commercially available kit (Boehringer Mannheim). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities, and total bilirubin concentrations in plasma were determined by routine clinical chemistry.

STATISTICAL ANALYSIS.

All data are given as mean values ± standard error of the mean. Statistical analysis between the experimental groups was performed using one-way ANOVA, followed by the Student-Newman-Keuls test (simvastatin experiment), or using the unpaired Student’s t-test (pravastatin and diosgenin experiments). Level of significance for all statistical analysis was set at P<0.05.

Table 1:

**PLASMA AND HEPATIC LIPID LEVELS AND BILIARY OUTPUT RATES IN CONTROL AND SIMVASTATIN-TREATED RATS.**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>rebound</th>
<th>simvastatin</th>
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<td><strong>plasma (mmol/l)</strong></td>
<td></td>
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<td>cholesterol</td>
<td>1.45 ± 0.12</td>
<td>1.52 ± 0.11</td>
<td>1.59 ± 0.18</td>
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<td>triglycerides</td>
<td>0.98 ± 0.11</td>
<td>0.40 ± 0.13a</td>
<td>0.36 ± 0.12a</td>
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<td><strong>liver (nmol/mg protein)</strong></td>
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<tr>
<td>total cholesterol</td>
<td>33.9 ± 2.43</td>
<td>45.9 ± 4.05ab</td>
<td>34.7 ± 2.36</td>
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<td>free cholesterol</td>
<td>29.7 ± 1.64</td>
<td>37.1 ± 2.90</td>
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<tr>
<td>cholesteryl esters</td>
<td>3.32 ± 0.73</td>
<td>9.29 ± 1.18ab</td>
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<tr>
<td>triglycerides</td>
<td>38.9 ± 5.40</td>
<td>34.2 ± 7.49</td>
<td>30.3 ± 3.48</td>
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<td>phospholipids</td>
<td>231 ± 7.2</td>
<td>235 ± 6.4</td>
<td>255 ± 6.9</td>
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<td><strong>biliary output (nmol·min⁻¹·100 g body weight)</strong></td>
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<td>bile salts</td>
<td>200 ± 30</td>
<td>227 ± 11</td>
<td>265 ± 29</td>
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<td>cholesterol</td>
<td>5.7 ± 0.63</td>
<td>21.5 ± 3.12ab</td>
<td>14.3 ± 1.91a</td>
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<tr>
<td>phospholipids</td>
<td>27.7 ± 5.9</td>
<td>53.3 ± 6.5a</td>
<td>62.9 ± 5.8a</td>
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</table>

Plasma and liver values represent the average ± SEM of 10 animals. Biliary output rates represent the average ± SEM of 3 animals.
a, significantly different from controls at P < 0.01,
b, significantly different from continuous at P < 0.01.
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RESULTS

CHARACTERISTICS OF SIMVASTATIN-TREATED RATS
We observed no significant differences in body weights or in the liver/body weight ratios between the control and simvastatin-treated groups (simvastatin-rebound [SR] and simvastatin-continuous [SC]; data not shown). During simvastatin-treatment, bile flow was slightly increased from 6.8 ± 0.9 µl·min⁻¹·100 g body weight⁻¹ in the control group to 8.1 ± 1.0 and 9.0 ± 1.8 µl·min⁻¹·100 g body weight⁻¹ in the SR and SC groups, respectively, but this did not reach statistical significance. This modest increase may be due to biliary secretion of simvastatin and/or its metabolites. No differences were found in either plasma ALT and AST activities or total bilirubin concentrations between the 3 experimental groups (data not shown).

EFFECTS OF SIMVASTATIN ON PLASMA, HEPATIC, AND BILIARY LIPIDS
Various parameters of lipid metabolism in control and simvastatin-treated rats are compared in Table 1. Plasma cholesterol concentrations were not affected in SR and SC rats, whereas the plasma triglyceride levels were significantly reduced to 40% and 37%, respectively, of control values. Continuous administration of simvastatin had no effect on the amounts of free cholesterol and cholesterylester in the liver. Withdrawal of simvastatin resulted in a significant increase of hepatic cholesterylester content to 280% of control values. Hepatic phospholipid and triglyceride levels were not significantly different between the three experimental groups. Simvastatin administration had no significant effects on biliary bile salt output. However, a profound effect on the biliary output rates of phospholipids and cholesterol was observed: simvastatin increased the phospholipid content of bile to 174% of control values in the SC group and to 172% in the SR group when expressed relative to bile salt content (Figure 1). Biliary cholesterol/bile salt ratios were elevated to 190% and 335% of control values in the SC and SR groups, respectively, whereas the cholesterol/phospholipid ratio was significantly increased in the SR group only, to 185% of control values (see Figure 1).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** RELATIVE BILE SALT, PHOSPHOLIPID AND CHOLESTEROL CONTENTS IN BILE OF CONTROL RATS AND SIMVASTATIN-TREATED RATS. Rats were fed standard chow (control, open bars), simvastatin-containing chow that was replaced 9-11 hours before the experiment (SR, black bars), or simvastatin-containing chow continuously (SC, gray). Bile was collected for 30 minutes as described in Experimental Procedures. Data are presented as ratios × 10³ of data presented in Table 1 and represent the average ± SEM of 3 animals. PL, phospholipids; BS, bile salts; CH, cholesterol. *: significantly different from control at p<0.01; ‡: significantly different from SC at p<0.01.
Effects of Simvastatin on mRNA Levels of ABC Transport Proteins in Liver

The relative mRNA levels of selected hepatic ABC transport proteins were analyzed by reverse-transcription PCR. We preferred reverse-transcription PCR analysis over Northern blotting because the latter technique is not sensitive enough to detect Mdr1a, Mdr1b, and Mrp1 transcripts in normal rat liver. Also, the specificity of the PCR-reactions can be confirmed by sequencing the PCR-products. Administration of simvastatin did not result in major changes of Mdr1a, Bsep, Mrp1, and Mrp2 mRNA levels (Figure 2). In contrast, Mdr2 mRNA levels in the SC group were strongly induced but were unaltered in the SR group. Mdr1b mRNA levels were increased in the SC and, and to a lesser extent, in SR groups.

Effects of Simvastatin on Protein Levels of ABC Transporters in Liver

Expression levels of various hepatic ABC transport proteins were examined by immunoblot analysis of crude liver membranes and canalicular membrane-enriched fractions. The results of experiment A are shown in Figure 3. All liver samples from the various experimental groups were tested in this way and similar results were obtained

Figure 2. MDR1A, MDR1B, MDR2, BSEP, MRP1, AND MRP2 mRNA EXPRESSION DURING SIMVASTATIN-TREATMENT. Lanes 1 and 2: control; lanes 3 and 4: simvastatin-rebound; lanes 5 and 6: simvastatin-continuous. mRNA was isolated from control rat liver or simvastatin-treated rat liver and 2.5 µg mRNA was transcribed into cDNA and subjected to PCR analysis as described in Experimental Procedures. Each band represents the results of a single animal. PCR-products are indicated at the left side of each gel, corresponding base pairs at the right side, and the experimental group above the figure. Representative experiment of n=5-7 per group.
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(n = 10 for each group). Continuous simvastatin administration resulted in pronounced increase of the C219 signal (detecting all Pgps) both in crude membrane pellets (Figure 3) and in canalicular membrane-enriched fractions (data not shown). Pgp levels remained elevated in the SR group. Mdr2 was increased in both the SC and SR groups (Figure 3). Bsep protein levels remained unchanged. A slight decrease of Mrp2 protein was observed in the SC group. Levels of dipeptidylpeptidase IV (DppIV) protein, used as a canalicular marker protein, were similar in all membrane fractions (data not shown).

LOCALIZATION OF ABC TRANSPORTERS IN LIVERS OF SIMVASTATIN-TREATED RATS

Immunohistochemical analysis of liver sections was performed to assess whether the simvastatin-induced expression of Mdr1b and Mdr2 resulted in changes in the distribution pattern of the encoded Pgps in liver. In control liver, the intensities of P3II-26 (detecting all Pgps) both in crude membrane pellets (Figure 3) and in canalicular membrane-enriched fractions (data not shown). Pgp levels remained elevated in the SR group. Mdr2 was increased in both the SC and SR groups (Figure 3). Bsep protein levels remained unchanged. A slight decrease of Mrp2 protein was observed in the SC group. Levels of dipeptidylpeptidase IV (DppIV) protein, used as a canalicular marker protein, were similar in all membrane fractions (data not shown).

Figure 3. Pgps, Bsep, Mrp2, and Mdr2 protein levels in membrane fractions of control rat liver and simvastatin-treated rat liver. Lanes 1 and 2: control; lanes 3 and 4: simvastatin-rebound; lanes 5 and 6: simvastatin-continuous. Livers from control rats and simvastatin-treated rats were used for the isolation of crude plasma membranes or canalicular membrane-enriched fractions; 100 μg of crude membrane proteins or 15 μg of canalicular-membrane enriched proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Immunoblotting analysis was performed using the primary antibodies C219, k12, k4 and k111, recognizing all Pgps, Mrp2, Bsep, or Mdr2, respectively. Bound antibodies were visualized as described in Experimental Procedures. For the analysis of Pgps, Bsep, and Mrp2, crude membrane protein fractions were used. Mdr2 could only be detected in canalicular-membrane protein-enriched fractions. Each band represents the results of a single animal. Transport proteins are indicated at the left side of each blot, apparent molecular weights at the right side, and the experimental group above the blot. Representative experiment of n=5-7/group.
Figure 4. Immunohistochemical localization of Mdr2 (A,B); C219 (C,D); and Bsep (E,F) in control rat liver (A,C,E) and simvastatin-treated rat liver (B,D,F). Frozen liver sections were stained with primary antibodies directed against Mdr2, all Pgps and Bsep, using P3II-26, C219 and k12 respectively. In normal rat liver, staining of Mdr2, although weak, was slightly stronger in periportal regions (a), as was the staining of C219 (c). In normal rat liver, Bsep protein was uniformly distributed across the liver acinus (e). In treated liver, the staining of Mdr2 (b) was predominantly increased in periportal areas. C219 staining of treated rat liver revealed a homogeneous staining throughout the liver lobule which was induced as compared with control rat liver (d). In treated rat liver, Bsep staining (f) was not affected as compared with control rat liver. P: portal area, C: central area. Typical staining patterns of n=4-6/group. Original magnification 20x.
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EFFECTS OF PRAVASTATIN AND DIOSGENIN ON BILE FORMATION AND MDR EXPRESSION

To study the specificity of the observed induction of Mdr2 and Mdr1b by simvastatin, experiments were performed with rats that received the hydrophilic HMG-CoA reductase inhibitor pravastatin according to the continuous protocol (pravastatin-continuous [PC]). In addition, another established model of biliary cholesterol hypersecretion, i.e., the diosgenin-fed rat, was included in the study. Pravastatin or diosgenin administration did not have significant effects on any of the animal characteristics described before. During pravastatin-treatment, however, bile flow was significantly increased from 7.1 ± 0.6 µl·min⁻¹·100 g body weight⁻¹ in the control group to 10.0 ± 0.7 µl·min⁻¹·100 g body weight⁻¹ in the PC group, most likely because of biliary excretion of pravastatin²⁶,³⁷. Diosgenin-feeding did not have an effect on bile flow. Plasma lipid levels and biliary lipid

Table 2:

<table>
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<tr>
<th></th>
<th>control</th>
<th>pravastatin continuous</th>
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<tbody>
<tr>
<td>plasma (mmol/l)</td>
<td></td>
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<tr>
<td>cholesterol</td>
<td>1.95 ± 0.07</td>
<td>1.78 ± 0.12</td>
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<tr>
<td>triglycerides</td>
<td>0.51 ± 0.06</td>
<td>0.46 ± 0.05</td>
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<td>biliary output (nmol·min⁻¹·100 g body weight)</td>
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<td>bile salts</td>
<td>256 ± 32</td>
<td>361 ± 39</td>
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<tr>
<td>cholesterol</td>
<td>4.6 ± 0.49</td>
<td>7.9 ± 0.87ᵃ</td>
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<tr>
<td>phospholipids</td>
<td>37.7 ± 3.0</td>
<td>62.7 ± 6.5ᵃ</td>
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*Plasma values represent the average ± SEM of 3 control and 5 pravastatin-treated animals. Biliary output rates represent the average ± SEM of 3 animals of each group.

³ᵃ, significantly different from controls at P<0.05.

Figure 5. RELATIVE BILE SALT, PHOSPHOLIPID AND CHOLESTEROL CONTENTS IN BILE OF CONTROL RATS AND PRAVASTATIN-TREATED RATS. Rats were fed standard chow (control, open bars) or pravastatin-containing chow (PC, black bars). Bile was collected for 30 minutes as described in Experimental Procedures. Data are presented as ratios × 10³ and represent the average ± SEM of 3 control and 5 pravastatin-treated animals. PL, phospholipids; BS, bile salts; CH, cholesterol.
output rates of control and pravastatin-treated animals are shown in Table 2. Plasma lipid levels were not significantly affected in the PC group compared with controls. Biliary output rates of bile salts as well as lipids were increased in the PC group compared with controls. However, when expressed relative to bile salt content, the biliary phospholipid and cholesterol contents in the PC group were not significantly increased (Figure 5), although both phospholipid and cholesterol output rates were clearly increased in treated animals (Table 2). In rats treated with diosgenin, biliary cholesterol output was stimulated about 6-fold, whereas phospholipid and bile salt secretion were not affected, as reflected in marked increases in cholesterol/bile salt and cholesterol/phospholipid ratios (Figure 6). Relative reverse-transcriptase PCR analysis of cDNA prepared from PC livers revealed an increase in Mdr2 and Mdr1b mRNA levels (Figure 7), albeit less evidently as observed in SC livers (Figure 2). Diosgenin treatment had no effect on mRNA levels of any of the tested proteins (data not shown).

DISCUSSION

The current study shows that statins increase the expression of Mdr2 and Mdr1b in rat liver. For its effects on Mdr2 expression, a continuous exposure to statins appeared to be necessary, because Mdr2 mRNA levels returned to control levels within 9-12 hours after simvastatin withdrawal. However, during this rebound phase, Mdr2 protein levels remained elevated and, accordingly, biliary phospholipid secretion was increased in both the SC and SR groups. In contrast, Mdr1b mRNA levels remained increased in the SR group, indicating different mechanisms of induction of Mdr2 and Mdr1b gene expression or differences in mRNA stability. Statin treatment did not affect plasma cholesterol levels in rats, but simvastatin drastically reduced plasma triglycerides, as expected. The effect on plasma triglycerides was sustained after withdrawal of simvastatin, probably indicating that hepatic very-low-density-lipoprotein production remained suppressed during the time course of this experiment. Neither liver size nor hepatic lipid content were affected by continuous statin feeding. In the simvastatin-rebound group, hepatic cholesterol and cholesterylester contents were strongly elevated, probably the result of an inappropriately high synthesis.
It is known that competitive inhibitors of HMG-CoA reductase strongly induce synthesis of HMG-CoA reductase while inhibiting its activity\(^8,38,39\). Simvastatin-administration did not affect Bsep expression and did not alter biliary bile salt output in either SC or SR groups. We observed a 2-fold increase in phospholipid output in simvastatin-treated animals concomitant with the increase in Mdr2 protein levels. These data clearly support the important role of Mdr2 in the regulation of biliary phospholipid secretion, as initially deduced from studies using mice with a disrupted Mdr2 gene\(^40\). Accordingly, biliary phospholipid secretion compared to that of bile salts is increased in mice in which the hepatic Mdr2 expression is induced by fibrate-feeding\(^41\) and in mice overexpressing MDR3, the human homologue of mdr2\(^42\). The finding that the biliary cholesterol/phospholipid ratios in SC and control groups are identical, despite suppression of cholesterol synthesis in the first group, suggests that phospholipid secretion per se is an important regulatory factor of cholesterol secretion. On the other hand, in the SR group cholesterol secretion is markedly enhanced relative to both bile salts and phospholipids. This indicates that, in this condition with an abrupt increase in de novo synthesis of cholesterol, the availability of excess cholesterol in the liver acts as an independent regulator of biliary cholesterol secretion.

To determine the specificity of the observed induction of Mdr2 and Mdr1b mRNA, the effects of pravastatin-administration on expression of these genes was also studied. In addition, a group of rats was fed diosgenin, representing another model of biliary cholesterol hypersecretion. In contrast to simvastatin, pravastatin treatment did not affect plasma triglycerides, indicating that, at the dosage used, simvastatin is a more potent inhibitor of sterol synthesis than pravastatin\(^43,44\). As a result, no profound changes in biliary lipid secretion compared with that of bile salts were observed, although there is a tendency towards stimulated biliary lipid secretion in PC animals. Also, a clear increase in Mdr2 and Mdr1b mRNA levels was detected in pravastatin-treated rats, suggesting similar cellular actions of simvastatin and pravastatin that are mediated via shared pathways. Data from diosgenin-treated rats demonstrate that hypersecretion of cholesterol can occur independently of Mdr2 induction and that cholesterol hypersecretion per se does not cause induction of Mdr2. In conclusion, our data suggest that the induction of Mdr2 expression, and, consequently, of biliary phospholipid secretion in statin-treated rats gives
rise to a proportional increase in cholesterol secretion. Cessation of statin treatment results in an additional stimulation of biliary cholesterol secretion that may be related to rapid expansion of the intracellular cholesterol pool because of a sudden increase in cholesterol synthesis.

Statins cause a transient state of cellular free cholesterol deprivation, resulting in enhanced transcription of sterol-regulated genes mediated by sterol regulatory element-binding proteins (SREBPs). SREBPs activate transcription by binding to sterol regulatory elements (SREs) in the promoter regions of target genes. Importantly, SREBP-1 is down-regulated and SREBP-2 is up-regulated in livers of hamsters and mice during treatment with lovastatin and a bile salt-binding resin. We hypothesize that transcriptional control of \textit{Mdr2} gene expression might, at least partially, be mediated via SREBPs. The 5'-flanking region of the \textit{Mdr2} gene contains elements that are possibly recognized by SREBPs: an inverted motif that is homologous to SRE-3 (position –278 to –269) and an inverted SRE-1 half site (position –193 to –188). The \textit{Mdr2}-positive cells were mainly localized to periportal regions of control livers; this was also observed in livers from simvastatin-treated animals. This zonal distribution is very similar to the reported distribution of HMG-CoA reductase and HMG-CoA synthase before and after statin-treatment, which suggests that the factors controlling the expression of \textit{Mdr2} and these enzymes may be similar. Staining with C219 of control rat liver revealed a similar heterogeneous staining pattern as observed for P3II-26. However, unlike P3II-26, the C219 signal was more homogeneously distributed in simvastatin-treated animals. Because the Bsep staining is unchanged and the \textit{Mdr2} staining is predominantly increased in periportal regions after simvastatin treatment, we conclude this may, at least partially, be due to increased expression of \textit{Mdr1b}-Pgp.

\textit{Mdr1b} mRNA levels also respond to statin administration, although this response is not as dramatic as observed in other experimental conditions. The induction of \textit{Mdr1b} mRNA persists after cessation of simvastatin treatment, in contrast to the situation observed for \textit{Mdr2}. This may be the result of a longer half-life of \textit{Mdr1b} mRNA, enhanced transcription rate, or a differential regulation of the expression of these 2 genes which is in accordance with the different physiological functions of these proteins. In rat liver, a direct induction of \textit{Mdr1b} gene expression has been demonstrated as a result of exposure to various xenobiotics, but the mechanism by which \textit{Mdr1b} mRNA is induced remains to be determined. Yamazaki et al. recently provided evidence that the biliary excretion of the relatively hydrophilic statin pravastatin is predominantly mediated by Mrp2. Whether Mrp2 is also involved in the biliary excretion of simvastatin or its metabolites is not known. Because of its hydrophobic nature, Mdr1a/1b may also be candidate transport proteins.

In conclusion, we have shown that during treatment of rats with the HMG-CoA reductase inhibitors simvastatin and pravastatin, the expression of \textit{Mdr2} and of \textit{Mdr1b} is enhanced, revealing a novel, until now unrecognized effect of these cholesterol-lowering agents. Increased \textit{Mdr2} expression may facilitate removal of excess cholesterol that enters the liver as a consequence of induced LDL receptor activity during statin-treatment. In addition, these findings may provide clues for mechanisms involved in regulation of Pgp expression and function in rat liver.
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