Cholesterol, bile acid and triglyceride metabolism intertwined
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Statins Increase Hepatic Cholesterol Synthesis and Stimulate Fecal Cholesterol Elimination in Mice

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**Introduction**

Statins represent the most commonly used class of drugs to treat and prevent cardiovascular diseases worldwide and supposedly work by decreasing cholesterol synthesis. In vitro statins act indeed as competitive inhibitors of HMG-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis. When statins are bound to HMGCR, they block the access of the natural substrate HMG-CoA to the catalytic site, and thereby interfere with cholesterol synthesis. As a result, the cholesterol content in the membrane of the endoplasmic reticulum (ER) decreases, causing activation of the SREBF Chaperone (SCAP), which then transports SREBP2 to the Golgi apparatus. In the Golgi apparatus, SREBP2 is activated via several cleavages and translocates to the nucleus. In the nucleus, the active SREBP2 can bind to the sterol regulatory element (SRE-1) of, among others, the low density lipoprotein receptor (LDLR) promoter and thereby induce expression of the LDLR. Increased number of LDLR on the cell membrane lead to decreased plasma LDL cholesterol levels explaining partly how LDL lowering is achieved upon statin treatment. This scenario seems a plausible sequel to reduced cholesterol synthesis, however, there is surprisingly scarce in vivo data available confirming that statins actually do reduce cholesterol synthesis rates. Cholesterol synthesis in vivo is mainly estimated via surrogate biomarkers e.g. intermediates of cholesterol synthesis. The concentration of mevalonic acid, squalene, cholestenol, lathosterol and desmosterol has indeed been shown to decrease in statin-treated patients. More direct measurements of cholesterol synthesis yielded controversial results. Sterol balance measurements in humans treated with pravastatin showed slight decreases in cholesterol synthesis. In contrast, Goldberg and coworkers found that cholesterol synthesis rates were unaltered in lovastatin-treated patients. Kallien et al. measured cholesterol synthesis in human subjects treated with pravastatin or placebo in a direct way using 13C-acetate, which is incorporated into the cholesterol molecule during cholesterol synthesis. They reported that cholesterol synthesis was even increased in pravastatin-treated individuals compared to controls. Furthermore, increased hepatic Hmgcr mRNA and enzyme activity levels have been observed in biopsies of humans as well as in various animal models treated with statins. Combined, these data challenge the current dogma that decreased cholesterol synthesis underlies the reduction of plasma cholesterol levels achieved by statin treatment. To address this challenge, we assessed the effects of statin treatment in detail using a mouse model. In order to study the effects of statins on cholesterol metabolism in detail, we selected three
different statins based on differences in their pharmacokinetic properties. Rosuvastatin is a hydrophilic statin which makes it more hepatoselective compared to atorvastatin and lovastatin, which are lipophilic.161 Atorvastatin and rosuvastatin have a longer half-life compared to lovastatin and next to that, lovastatin is a prodrug i.e. it needs to be activated in the enterohepatic circulation to become active.161,162 In our study, statin treatment led to a slight decrease in plasma cholesterol levels. Paradoxically, cholesterol synthesis rates were robustly increased upon statin treatment in sharp contrast with what would be expected upon HMGCR inhibition.

Materials and Methods

Animals and treatments

Male C57BL/6J mice were purchased from Charles River (L’Arbresle, Cedex, France) and housed under standard conditions with ad libitum access to food and water, under a 12-hour light/12-hour dark cycle in a temperature-controlled environment. Mice were fed a standard rodent chow diet (RMH-B, AB-Diets, Woerden, The Netherlands), containing 0.023% cholesterol, with or without the supplementation of 0.01% rosuvastatin (Crestor, AstraZeneca, Zoetermeer, The Netherlands), 0.05% atorvastatin (Lipitor, Pfizer, Freiburg, Germany) or 0.2% lovastatin (1 A Pharma, Oberhaching, Germany) for 2 weeks (n=7 per group). These doses were chosen based on previous publications for use in mice without any toxic side effects.31,160,166 The institutional ethical committee for animal experiments of the University of Groningen approved all experimental procedures.

Experimental Procedures

After 5 days of statin treatment, mice were injected intravenously with D2-cholesterol and orally gavaged with D1-cholesterol; blood spots were taken from the tail vein at 0, 3, 6, 12, 24, 48, 72, 92, 120, 144, 168, 192 hours after administration of the stably labelled isotopes, to measure the absorption of cholesterol.166 To assess cholesterol synthesis, 2% 13C-acetate was added to the drinking water ad libitum from day 12 on for 72 hours. The 13C-acetate addition started at 9 a.m. and bloodspots were taken at time points 0, 24, 31, 48 and 57 hours. Feces were collected during 24 hours prior to termination. At the end of the experiment, bile cannulations were performed and bile was collected continuously for 30 minutes as described by Brufau et al.166 Mice were terminated under anesthesia by cardiac puncture, and consecutively the liver and the small intestine were excised. After excision, the liver was weighed and snap frozen in liquid N2.

Analysis of cholesterol fluxes

After extraction of fecal neutral sterols and biliary cholesterol, the determination of the concentrations was carried out by GC (HP 6890 series, Hewlett Packard) and GCMS (5975C inert XL EI/CI MSD and 7890A GC system, Agilent Technologies) as described previously.25,166 Fractional absorption and transintestinal cholesterol excretion were calculated according to previously published protocols.25,166 Fecal bile salts were measured from the acid sterol fraction of the feces after extraction of neutral sterols, by GC as detailed elsewhere.81

Mass Isotopomer Distribution Analysis of cholesterol synthesis using the 13C-acetate method

Fractional cholesterol synthesis was determined by Mass Isotopomer Distribution Analysis (MIDA) using 13C-acetate (Isotec, Miamisburg, OH, USA) as labeled precursor according to the method originally published by Hellerstein.168 The incorporation of 13C into newly synthesized cholesterol molecules was quantified by GCMS (5975C inert XL EI/CI MSD and 7890A GC system, Agilent Technologies). Using these data, cholesterol synthesis was calculated as detailed elsewhere.25 Briefly, cholesterol was extracted from blood spots, taken at the time points indicated above, using 1 ml 95% ethanol/acetone (1:1, v/v) and trimethylsilylated using N,O-bis-(trimethyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) overnight at room temperature. Cholesterol label enrichment was determined by capillary gas chromatography on a Agilent gas chromatograph (7890A; Amstelveen, The Netherlands) connected to a Agilent mass spectrometer (5975C). Isotope ratios were determined in the selected ion monitoring mode on m/z 458 (M+), 465 (M+7) and corrected for the fractional distribution due to natural abundance of 13C by multiple linear regression as described by Lee et al.166 to obtain excess fractional distribution of mass isotopomers. M+ and M+7 were used in MIDA calculations to determine the fraction of newly synthesized cholesterol. Precursor pool enrichment was estimated from the
ratios of $M_3$ and $M_5$ based on theoretical multinomial curves. Next, from a curve in which $M_3$ and $M_5$ are plotted against precursor pool enrichment, mass-isotopomer distributions of the newly synthesized cholesterol can be estimated ($M_{3\text{new}}$ and $M_{5\text{new}}$). The ratio of the measured fractional distribution of cholesterol and the estimated fractional distribution in the newly synthesized cholesterol gives the fractional contribution of newly synthesized in the cholesterol pool ($f_{\text{new}}$).

\[
(f_{\text{new}})_t = \frac{(M_{3\text{new}})_t}{(M_{1\text{new}})_t} - \frac{(M_{5\text{new}})_t}{(M_{3\text{new}})_t}
\]

**Plasma and hepatic lipid analysis**

Plasma cholesterol was measured using an enzymatic kit (DiaSys Diagnostic Systems, Holzheim, Germany) and the same assay was used to measure hepatic cholesterol after extraction of cholesterol from liver homogenates according to the method of Bligh & Dyer.\(^{112}\) Separation of the lipoproteins was performed with fast protein liquid chromatography (FPLC) using a Superose 6 10/300 GL column (GE Healthcare, Uppsala, Sweden) as described.\(^{170}\) Triglycerides in the lipoprotein fractions were measured using a commercially available kit (Roche Diagnostics, Mannheim, Germany).

**Targeted quantitative proteomics**

Targeted proteomics was used to quantify HMGCR in homogenized liver tissue via the isotopically labeled peptide standard FLSDAEIQLVNAK (containing 13C-labeled lysine), derived from synthetic peptide concatamers (QconCAT) (PolyQuant GmbH, Germany). Briefly, homogenized tissues (50 μg protein) were mixed with 3.22 fmol of QconCAT per 1 μg of total protein. In-gel tryptic digestion (1:100 g/g sequencing grade modified trypsin V5111, Promega) was performed after reduction with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide proteins, followed by solid phase extraction (SPE C18-Aq 50 mg/1mL, Gracepure) for sample cleanup. The peptide was targeted and analyzed by a triple quadrupole mass spectrometer (MS) equipped with a nano-electrospray ion source (TSQ Vantage, Thermo Scientific). The chromatographic separation of the peptides was performed by liquid chromatography on a nano-UHPLC system (Ultimate UHPLC focused, Dionex) using a nano column (Acclaim PepMap100 C18, 75 μmx500mm 2μm, 100 Å) with a linear gradient from 3-60 % v/v acetonitrile plus 0.1% v/v formic acid in 110 minutes at a flowrate of 200 nL/min. The MS traces were manually curated using the Skyline software\(^{171}\) prior to integration of the peak areas for quantification. The sum of all transition peak areas for the endogenous peptide and isotopically labeled QconCAT-peptide standard was used to calculate the ratio between the endogenous and standard peptides. The concentrations of the endogenous peptides were calculated from the known concentration of the standard and expressed in fmol/μg of total protein.

**Liver samples**

Liver samples were homogenized in PBS and sonicated to a 15 (w/w%) liver homogenate. To allow quantification of 3-hydroxy-3-methylglutaryl (HMG), an internal standard of 0.05 mg/mL 4-phenyl-butrylic acid was added to 1.5 mL homogenate and centrifuged at 20800 g for 5 minutes at 4 °C (Eppendorf Centrifuge 5417R) and the supernatant was collected. 100 μL ethoxim was added to the samples and subsequently incubated at 60 °C. Around 150 μL of 12N HCl and around 10 mg of NaCl were added to the samples. Organic acids were extracted by adding 4 mL of a mixture of ether and ethylacetate (1:1), vortexed for 3 minutes and centrifuged for 10 minutes at 3500 g (MegaFuge 1.0, Heraeus Sepatech). The supernatant was collected and the extraction was repeated once more. The supernatant was evaporated at 50 °C under a stream of nitrogen. The dried samples were derivatized by adding 100 μL BSTFA:TMCS:Pyridine (5:0.06:1) and heated for 30 minutes at 60 °C. Concentrations of HMG were further analyzed using GCMS (Finigan Trace GCMS, ThermoQuest) as previously described.\(^{172}\)

**Analysis of hepatic 3-hydroxy-3-methylglutaryl levels**

Liver samples were homogenized in PBS and sonicated to a 15 (w/w%) liver homogenate. To allow quantification of 3-hydroxy-3-methylglutaryl (HMG), an internal standard of 0.05 mg/mL 4-phenyl-butrylic acid was added to 1.5 mL homogenate and centrifuged at 20800 g for 5 minutes at 4 °C (Eppendorf Centrifuge 5417R) and the supernatant was collected. 100 μL ethoxim was added to the samples and subsequently incubated at 60 °C. Around 150 μL of 12N HCl and around 10 mg of NaCl were added to the samples. Organic acids were extracted by adding 4 mL of a mixture of ether and ethylacetate (1:1), vortexed for 3 minutes and centrifuged for 10 minutes at 3500 g (MegaFuge 1.0, Heraeus Sepatech). The supernatant was collected and the extraction was repeated once more. The supernatant was evaporated at 50 °C under a stream of nitrogen. The dried samples were derivatized by adding 100 μL BSTFA:TMCS:Pyridine (5:0.06:1) and heated for 30 minutes at 60 °C. Concentrations of HMG were further analyzed using GCMS (Finigan Trace GCMS, ThermoQuest) as previously described.\(^{172}\)

**Analysis of lathosterol, desmosterol and lanosterol in plasma**

To allow quantification of lathosterol, desmosterol and lanosterol in plasma, 100 μL internal standards of D $_3$-β-sitosterol (22.8 μmol/L) and 100 μL D $_3$ -lathosterol (3.8 μmol/L) were added to empty tubes and evaporated at 100 μL internal standards of D$_6$-β-sitosterol (22.8 μmol/L) and 100 μL D$_3$ -lathosterol (3.8 μmol/L) were added to empty tubes and evaporated at 40 °C under a stream of nitrogen. Subsequently, the samples were added to the tubes and extraction was performed as described previously.\(^{173}\) After extraction, lathosterol, desmosterol and lanosterol concentrations were further analyzed using GCMS (Agilent 5973N) as previously described.\(^{25}\)

**Table 1. Basal parameters, plasma and hepatic total cholesterol after 2 weeks of statin treatment**

<table>
<thead>
<tr>
<th>Statin Treatment</th>
<th>Control</th>
<th>Rosuvastatin</th>
<th>Atorvastatin</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.9 (25.5-28.4)</td>
<td>26.7 (26.1-27.1)</td>
<td>25.6 (24.7-26.1)</td>
<td>24.4 (23.5-25.1)*</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>4.3 (3.7-5.0)</td>
<td>4.3 (3.6-4.8)</td>
<td>5.4 (4.4-6.5)</td>
<td>4.1 (3.7-4.6)</td>
</tr>
<tr>
<td>Fecal Output (g/day)</td>
<td>0.77 (0.73-0.83)</td>
<td>0.85 (0.81-0.85)</td>
<td>0.79 (0.65-0.89)</td>
<td>0.60 (0.52-0.75)*</td>
</tr>
<tr>
<td>Liver Weight (% of BW)</td>
<td>4.6 (3.6-5.0)</td>
<td>4.9 (4.6-5.0)</td>
<td>4.4 (4.4-4.9)</td>
<td>5.1 (5.0-5.6)*</td>
</tr>
<tr>
<td>Plasma Total Cholesterol (nmol/l)</td>
<td>2.32 (2.03-2.49)</td>
<td>2.00 (1.86-2.26)</td>
<td>1.72 (1.38-1.95)*</td>
<td>1.81 (1.64-1.95)*</td>
</tr>
<tr>
<td>Hepatic Total Cholesterol (nmol/mg protein)</td>
<td>29.7 (28.1-31.0)</td>
<td>29.2 (26.9-31.2)</td>
<td>31.5 (28.3-34.0)</td>
<td>38.5 (34.8-41.0)*</td>
</tr>
</tbody>
</table>

Values are presented as median with the interquartile range, * p<0.05 vs. control.
Analysis of gene expressions
RNA isolation, cDNA synthesis and real time quantitative PCR were used to analyze gene expression and were performed as described previously.\(^{115}\)

Statistics
Statistical analysis was performed using the Kruskal-Wallis H test followed by Conover post hoc comparisons in Brightstat.\(^{116}\) Values are presented as XY plots and Tukey box plots. P-values <0.05 were considered statistically significant with n=7 animals per group.

Results

Statins decrease plasma cholesterol in mice
Two weeks of treatment of mice with rosvastatin, atorvastatin or lovastatin was well tolerated, only changes in body weight, liver weight and mass fecal output were observed in lovastatin-treated mice (Table 1). Plasma total cholesterol decreased in atorvastatin and lovastatin treated mice mainly attributable to decreases in HDL as indicated by FPLC profiles (Figure 1). Furthermore, hepatic total cholesterol content was somewhat increased in lovastatin-treated mice only (Table 1).

Statins paradoxically increase cholesterol synthesis, primarily in the liver
Since statins are supposedly effective by inhibiting HMGCR, we next evaluated the impact of statin administration on cholesterol synthesis. To assess in vivo cholesterol synthesis rates in response to statins, \(^{13}\)C-acetate was added to the drinking water of the mice. Remarkably and in apparent contrast with the expectation of statins inhibiting HMGCR activity, treatment with atorvastatin and lovastatin led to a robust increase in measured cholesterol synthesis rates (Figure 2A). These data reflect cholesterol synthesis during a period of 3 days. To investigate the contribution of the liver or small intestine, organ-specific cholesterol synthesis in these organs was determined using deuterated water. Also, certain limitations associated with the use of labeled acetate as a precursor to measure cholesterol synthesis have been reported, likely related to differences in cell entry of acetate or its conversion into acetyl-CoA between different cells and organs.\(^{174}\) Labeled water equilibrates with body water within minutes and therefore doesn’t have this potential drawback. Therefore cholesterol synthesis measured using D\(_2\)O served not only as organ-specific assessment this parameter, but also as a validation of the results obtained with \(^{13}\)C-acetate. The data obtained using D\(_2\)O confirmed that statin treatment strongly enhances cholesterol synthesis. A strong induction was observed in the liver, while only a modest increase was observed in the proximal, middle and distal parts of the small intestine (Figure 2B). Increased hepatic cholesterol synthesis thus appears to

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**Figure 1: Lipoprotein profile in plasma analyzed by FPLC.**
Total cholesterol concentrations were measured in FPLC fractions of 0.5 mL.

**Figure 2: Cholesterol synthesis is increased in statin-treated mice.**
(A) increased total body cholesterol synthesis measured by the incorporation of \(^{13}\)C-acetate into the cholesterol molecule over a period of 72 hours. (B) Hepatic cholesterol synthesis is highly increased after statin treatment, compared to the proximal, middle and distal parts of the small intestine using deuterium water. (C) mRNA levels of Hmgcr in the liver are increased, however not in the small intestine. (D) Hepatic HMGCR protein concentrations were increased after statin treatment.
be responsible for a major part of the increase in cholesterol synthesis found with the \(^{13}\text{C}-\text{acetate method}. In keeping, no increase in gene expression of Hmgcr in the small intestine was detected in contrast to the liver, further indicating that statins induced hepatic cholesterol synthesis stronger than intestinal cholesterol synthesis (Figure 2C). Because HMGCR is subject to extensive regulation at the protein level, hepatic protein levels of this enzyme were measured as well. Indicative of post-transcriptional regulation as mechanism counteracting inhibition of HMGCR by statins, hepatic protein levels showed a stronger induction as mRNA levels. On average, HMGCR protein levels were 6-, 15- and 11-fold higher in mice treated with rosuvastatin, atorvastatin and lovastatin, respectively, compared to controls (Figure 2D).

Statin treatment results in accumulation of HMG

Apparently, an inhibitor of HMGCR is able to increase cholesterol synthesis and this led to the hypothesis whether an accumulation of the natural substrate of HMGCR could outcompete the binding of statins to HMGCR. We did not succeed in measuring HMG-CoA, instead, concentrations of HMG, a degradation product of HMG-CoA, were analyzed. Hepatic HMG levels were indeed increased in statin-treated mice (Figure 3A), suggesting that there is an accumulation of the natural substrate for HMGCR that may counteract the inhibitory action of the statins to a certain degree and thereby contribute to the observed increase in cholesterol synthesis. Next, the effects of statins on the cholesterol synthesis pathway downstream of HMGCR was investigated. Statin treatment leads to an increased expression of the regulator of cholesterol synthesis Srebp2 in the liver (Figure 3B). Subsequently, gene expression levels of other enzymes in the cholesterol synthesis pathway, which are target genes of SREBP2, were analyzed. Statin treatment strongly induced gene expression of the cholesterol synthesis enzymes Mok (mevalonate kinase), PmeK (phosphomevalonate kinase), Fdft1/Sqs (Farnesyl-diphosphate farnesyltransferase 1) and Sqle (squalene epoxidase) (Figure 3B).

Intermediates of cholesterol synthesis do not reflect synthesis rates in statin-treated mice

Intermediates of cholesterol synthesis are often used to estimate cholesterol synthesis and these data indicate that a decrease in cholesterol synthesis by statins can be monitored by a reduction in the plasma levels of precursors of cholesterol synthesis. The concentration of the cholesterol synthesis precursors lathosterol, lanosterol and desmosterol were analyzed in plasma to determine whether these are indeed suitable as surrogate biomarkers for cholesterol synthesis. Lathosterol measurements showed a slight increase atorvastatin- and lovastatin- treated mice. A minor increase in lanosterol levels was observed only in lovastatin-treated mice. No differences in desmosterol concentrations could be detected in any of the groups (Figure 3C). Altogether, none of these markers accurately reflected cholesterol synthesis rates in the presence of statins.
**Fecal cholesterol excretion is increased in statin-treated mice**

To investigate via which pathway the flux of cholesterol from the plasma is mediated, we analyzed gene expressions of Srebp2 target genes which mediate cholesterol in- and efflux transport. The LDL receptor is a transporter which mediates import of cholesterol into the cell, and the heterodimer of ABCG5 (ATP-binding cassette, sub-family G (WHITE), member 5) and ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8) regulates cholesterol export. In the liver, statin treatment increased Ldlr expression, which suggests enhanced hepatic uptake of cholesterol from the plasma (Table 2) as well as Abcg5 and Abcg8 expression, suggesting increased transport of cholesterol from the liver into the bile (Table 2). Similarly, these transporters are also expressed in the small intestine where LDLR can mediate the influx of cholesterol from the basolateral side of the enterocytes, while the ABCG5/ABCG8 heterodimer facilitates efflux towards the intestinal lumen. In contrast to the liver, no increase in gene expression levels of any of these transporters was detected in the small intestine (Table 2). The results described above showed that statins enhance cholesterol synthesis but do not increase plasma cholesterol levels or to a major extent hepatic cholesterol content. We therefore hypothesized that the newly synthesized cholesterol is quickly eliminated from the body and thus assessed the effect of statins on fecal neutral sterol excretion. Indeed, fecal neutral sterol excretion was increased in the statin-treated mice (Figure 4A). Fecal cholesterol can originate from three different pathways i.e.: (i) unabsorbed cholesterol from the diet, (ii) biliary cholesterol secretion or (iii) transintestinal cholesterol excretion (TICE). In addition, absorption of cholesterol in the small intestine is also an important determinant of the cholesterol balance. The intake of cholesterol via the diet was the same in all groups (Figure 4B) and fractional cholesterol absorption was decreased only in lovastatin-treated mice (Figure 4C). The dietary cholesterol and fractional absorption alone are not sufficient to explain the increase in fecal neutral sterols. However, biliary cholesterol was increased upon statin treatment and this was most pronounced in the

![Figure 4: Atorvastatin and lovastatin treatment increases fecal neutral sterol excretion.](image)

(A) fecal neutral sterols are increased. The increase of fecal neutral sterols could not be explained by changes in (B) dietary cholesterol or (C) fractional cholesterol absorption but is largely due to the contribution of (D) biliary cholesterol and slightly to (E) TICE.

| Table 2. Gene expression levels in liver and proximal small intestine |
|----------------------|---------|---------|---------|---------|
|                     | Control | Rosuvastatin | Atorvastatin | Lovastatin |
| **Liver**           |         |           |           |          |
| Abcg5               | 1.0 (0.6-1.6) | 1.5 (1.3-1.7) | 2.9 (1.9-3.6)* | 4.2 (3.0-5.0)* |
| Abcg8               | 1.0 (0.5-1.5) | 1.5 (1.3-1.7) | 2.9 (1.9-3.8)* | 3.6 (2.6-4.4)* |
| Ldlr                | 1.0 (0.8-1.2) | 1.3 (1.2-1.4)* | 2.1 (1.5-2.7)* | 1.9 (1.5-2.4)* |
| **Proximal Small Intestine** |         |           |           |          |
| Abcg5               | 1.0 (0.6-1.7) | 0.7 (0.2-0.9) | 0.9 (0.7-1.0) | 0.3 (0.2-0.5)* |
| Abcg8               | 1.0 (0.5-1.8) | 0.9 (0.1-1.3) | 1.0 (0.8-1.2) | 0.4 (0.2-0.5) |
| Ldlr                | 1.0 (0.7-1.2) | 0.6 (0.5-0.8)* | 0.8 (0.5-1.1) | 0.4 (0.3-0.5)* |

Results were normalized to 36b4 mRNA levels and to the values of the control group. Values are expressed as median with the interquartile range, *p<0.05 vs. control.
lovastatin-treated group (Figure 4D). By Subtracting non-absorbed cholesterol of dietary and biliary origin from the fecal neutral sterols, the contribution of TICE can be estimated. Interestingly, statin treatment led to increased TICE only in atorvastatin-treated mice (Figure 4E). These results show that the increased fecal neutral sterol excretion is mainly due to increased excretion of cholesterol via the hepatobiliary route upon administration of rosuvastatin and lovastatin, while atorvastatin leads to a substantial increase in TICE.

Bile salt synthesis is a major pathway of cholesterol catabolism. Therefore, the effects of statin treatment on hepatic mRNA expression of the key genes in bile salt synthesis were measured. Hepatic expression of cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1) was somewhat higher in the animals treated with atorvastatin, but was unaffected in mice receiving rosuvastatin or lovastatin (Figure 5A). Cytochrome P450, family 8, subfamily b, polypeptide 1 (Cyp8b1) was significantly increased in atorvastatin mice only (Figure 5A). Under steady-state conditions, fecal bile salt excretion reflects bile salt synthesis. Indicative of increased bile salt synthesis, fecal bile salts were increased in mice treated with atorvastatin whereas no differences were observed in mice receiving the other statins (Figure 5B). The increase in fecal bile salt secretion in atorvastatin-treated mice could be entirely attributed to cholate-derived bile salt species (data not shown). Increased cholesterol catabolism due to increased bile salt synthesis therefore likely underlies the increased cholesterol synthesis observed in atorvastatin-treated mice to some extent. However, bile salt synthesis does not appear to be affected in mice treated with rosuvastatin or lovastatin and therefore cannot explain increased cholesterol synthesis in those mice.

Discussion

This study demonstrates that treatment of mice with statins paradoxically increases cholesterol synthesis. These findings are surprising considering the fact that statins inhibit HMGCR, the rate-limiting enzyme of cholesterol synthesis. The three statins used in this study induced different effects. Atorvastatin and lovastatin strongly increased cholesterol synthesis whereas only a slight effect of rosuvastatin was observed. Surprisingly, the effect on plasma cholesterol was opposite. Despite increasing cholesterol synthesis, atorvastatin and lovastatin modestly decreased levels of plasma cholesterol, confirming earlier studies. The reduced efficacy of statins in rodents compared to humans has been explained by the fact that statins primarily control levels of LDL cholesterol which is compared to HDL a minor component in plasma lipoproteins of mice.177

The inhibition of cholesterol synthesis by statins has been described in numerous cell culture studies.155,178 Assuming that this effect can be translated to in vivo conditions, cholesterol synthesis is rarely measured in experiments in humans or rodent models. Yet, compared to the in vitro experiments in cultured cells, the in vivo situation is more complex because due to the longer term more systems adaptation can be expected. It is well-known that statins strongly induce expression of Hmgcr via a SREBP2-mediated pathway.179 Although in our experiments statins were dosed via the diet at high levels, in principle fasting during the light period could lead to a decrease in statin concentration inducing a short relieve of inhibition leading to an overshoot of synthesis. An overshoot of cholesterol synthesis upon statin withdrawal has been shown in rats.156,179 To investigate whether such a phenomenon occurred during our experiments, we have monitored cholesterol synthesis for up to 72 hours. Surprisingly synthesis rates were lowest during the light phase indicating that fasting did not induce overshoots in synthesis. In contrast, apart from the well-known circadian rhythm in cholesterol synthesis,180 rates were remarkably constant. Measurements of organ-specific cholesterol synthesis in this study revealed that hepatic cholesterol synthesis was profoundly increased in statin-treated animals following the extent of overexpression of the enzyme. Intestinal synthesis was increased much less. These results are in accordance with the study of Chuang et al. who have measured an increase in acute cholesterol synthesis in BALB/c mice treated with simvastatin.181 Interestingly, Freeman et al. showed that lovastatin reduced HMGCR activity in human intestinal biopsies.182 Administration of compounds that inhibit in vivo processes usually induce adaptive homeostatic reactions. It is, however, difficult to predict to what extent such compensatory reactions of a system could be counterbalanced by adjustment of the dose of the inhibitor. Since statins are competitive inhibitors, a compensatory increase in the amount of HMG-CoA reductase protein or accumulation of the endogenous substrate, HMG-CoA, could impact the competition balance and cholesterol synthesis rates. HMG-CoA protein levels were up to 15-fold higher in statin-treated
mice. We have not succeeded in measuring hepatic levels of HMG-CoA. As a proxy of increased HMG-CoA we have measured the concentration of the breakdown product HMG which was indeed increased. Those data suggest that the competition hypothesis may be valid. The increased cholesterol synthesis was not reflected by increased cholesterol in plasma or in the liver indicating that steroid produced in excess is possibly removed by reverse cholesterol transport. Indeed, statin treatment stimulated fecal neutral sterol excretion. The increase in fecal neutral sterol excretion could not be explained by changes in dietary cholesterol intake or fractional cholesterol absorption. Calculation of cholesterol fluxes revealed that the increase in neutral sterols after statin treatment is primarily caused via induction of biliary cholesterol secretion. This is in accordance with data from studies in statin-treated rats after statin treatment is primarily caused via induction of biliary cholesterol secretion.

Interestingly, the increase in neutral sterols observed in atorvastatin-treated mice was also due to an increase in TICE. No significant induction of TICE in lovastatin-treated mice was observed in our study, in contrast to a previous study from Le May et al. who did observe stimulated TICE in lovastatin-treated mice. We have no explanation for this discrepancy.

Cholesterol synthesis is rarely measured directly, in most cases synthesis is estimated from surrogate markers such as intermediates of the cholesterol synthesis pathway. The plasma concentrations of these intermediates such as lathosterol, lanosterol or desmosterol are generally accepted as suitable surrogate markers for cholesterol synthesis. It is mostly not appreciated that the validity of the use of this surrogate markers depends on constancy of the ratios of activities of the involved enzymes. Statins do not only lead to induction of HMGCR but via the Srebp2 pathway also induce expression of the other enzymes in the pathway. Increased activity of these enzymes will invalidate the use of the concentration of their substrates or products as marker of the rate of cholesterol synthesis. Indeed we saw little change in the plasma concentration of lathosterol, desmosterol and lanosterol at the increased rates of cholesterol synthesis measured in this study. Clearly these surrogate markers should be used with great caution as biomarkers of cholesterol synthesis in patients treated with statins.

This research contributes to a better understanding of the mechanism of action of one of the most prescribed drugs in the world. Translation of the results to humans will require in depth estimation of cholesterol synthesis in humans which is difficult to accomplish in humans because of ethical reasons. It should be noted that there are certain species differences in cholesterol metabolism upon statin treatment. For instance, in line with data reported previously, we found increased biliary cholesterol secretion in statin-treated mice. However, a reduction of biliary cholesterol secretion and saturation index has been observed in humans treated with pravastatin or lovastatin. In addition, statin use was associated with a reduction in the risk of gallstone disease, supporting decreased rather than increased biliary cholesterol secretion rates. The effect of statins on TICE has not been assessed in humans. Augmented fecal bile salt excretion, a surrogate of synthesis, has been reported in atorvastatin-treated mice. We also observed increased fecal excretion of bile salts in mice treated with atorvastatin, but found no change with other statins. In humans, lovastatin treatment was associated with a decrease in bile salt synthesis. The effects on biliary secretion may indicate inhibition of hepatic cholesterol synthesis in humans. Yet, the few early studies in statin-treated humans that applied direct methods to measure cholesterol synthesis and failed to prove a reduced cholesterol synthesis or even pointed towards increased cholesterol synthesis indicate that whole body cholesterol synthesis in humans may also be augmented in humans. If increased cholesterol synthesis after statin treatment is also the case in humans, than it would be important to investigate whether this would also lead to enhanced fecal excretion of neutral sterols or if the newly synthesized cholesterol undergoes a different fate.

In summary, this study reveals a paradoxical increase in hepatic cholesterol synthesis in mice treated with the HMG-CoA reductase inhibitors rosuvastatin, atorvastatin and lovastatin. However, statin-treated mice do have reduced plasma cholesterol levels. This can likely be explained by increased fecal neutral sterol excretion, which is mainly attributed to an increase in the biliary cholesterol flux in rosuvastatin- and lovastatin-treated mice. After atorvastatin treatment TICE also contributes to fecal neutral sterol excretion. In addition, this study is also the first to question the clinical usefulness of cholesterol precursor levels in plasma as surrogate biomarkers for cholesterol synthesis.

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