Atherosclerosis and Lipoproteins

Studies on the Cholesterol-Free Mouse

Strong Activation of LXR-Regulated Hepatic Genes When Replacing Cholesterol With Desmosterol

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Objective—Characterization of cholesterol homeostasis in male mice with a genetic inactivation of 3β-hydroxy-Δ24-reductase, causing replacement of almost all cholesterol with desmosterol.

Methods and Results—There was an increase in hepatic sterol synthesis and markedly increased fecal loss of neutral sterols. Fecal excretion of bile acids was similar in knockout mice and in controls. The composition of bile acids was changed, with reduced formation of cholic acid. It was shown that both Cyp7a1 and Cyp27a1 are active toward desmosterol, consistent with the formation of normal bile acids from this steroid. The levels of plant sterols were markedly reduced. Hepatic mRNA levels of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase, Srebp-1c, Srebp-2, Cyp7a1, Abcg5, Abcg8, and Fas were all significantly increased.

Conclusions—The changes in hepatic mRNA levels in combination with increased biliary and fecal excretion of neutral steroids, reduced tissue levels of plant sterols, increased plasma levels of triglyceride-rich VLDL, are consistent with a strong activation of LXR-targeted genes. The markedly increased fecal loss of neutral steroids may explain the fact that the Dhcr24−/− mice do not accumulate dietary cholesterol. The study illustrates the importance of the integrity of the cholesterol structure—presence of a double bond in the steroid side-chain is compatible with life but is associated with serious disturbances in sterol homeostasis. (Arterioscler Thromb Vasc Biol. 2007;27:2191-2197.)

Key Words: desmosterol ■ sterol deficiency ■ sterol malabsorption ■ LXR-activation ■ Abcg5

In a previous work we generated mice with a disruption of 3β-hydroxy-Δ24-reductase (desmosterol reductase, Dhcr24), as a result of which cholesterol synthesis is blocked because of absence of reduction of the C24–C25 double bond.1 Surprisingly, mice surviving to full term were viable and had no gross morphological defects. There were, however, abnormalities in male gonads and in oocyte maturation, apparent reduction of subcutaneous and mesenterial fat, apparent decrease of myelination of the central nervous system, and hypotonia. Because the Dhcr24 knockout mice are unable to reproduce, they have to be continuously generated from the corresponding heterozygotes. The relatively mild phenotype in marked contrast to that of the human desmosterolosis patients, who have multiple severe inborn defects.2,3 A plausible reason for the difference could be the availability of maternal cholesterol during mouse embryonic development.1

In the adult knockout mouse, desmosterol exceeded cholesterol on a molar basis by 80-fold in the plasma and by 15-fold in the brain. In addition, both brain and plasma displayed about 25% and 40% reduction of total steroids, respectively. Cholesterol, desmosterol, and total sterol levels in heterozygous mice were identical to those of the wild-type.

It was considered to be of interest to characterize cholesterol homeostasis in the Dhcr24 knockout mice, in particular to elucidate the mechanism behind the sterol depletion and the metabolic consequences of this.

The extra double bond present in the side chain of desmosterol makes the molecule more polar and affects its interaction with phospholipids. It has been shown that the efflux of biosynthetic desmosterol from cells is 3 times more efficient than that of cholesterol.4 This would be expected to affect the membrane properties of the steroid and also its potential to suppress sterol synthesis. On the other hand, it is known that desmosterol can be sensed by SCAP and induce a conformational change similar to that caused by cholesterol.5

Since a Δ24 double bond is introduced in connection with normal bile acid biosynthesis, it is possible that normal bile acids are formed from desmosterol. Whether desmosterol...
may serve as a substrate in the classical pathway to bile acids starting with a 7α-hydroxylation or in the alternative pathway starting with a 27-hydroxylation is not known.

Very recently it was reported that desmosterol is able to activate LXR in vitro. If this effect is of importance also under in vivo conditions, one would expect activation of LXR-targeted genes in our animal model.

All the mice studied in the present work were generated in 2003 in Quark Biotech Inc. Some studies on cholesterol homeostasis in the brain of these animals have been reported. More recent attempts to get Dhcr24−/− progeny from the heterozygous breeders in Switzerland, Japan, in our Swedish laboratory as well as in Quark Biotech have not been successful, and only very few mice have survived more than a few days or weeks. The few survivors in the later generations had, however, the same phenotype as the first generations with the same composition of steroids. Why the first generation of mice survived for a longer time than latter generations has not been clarified. The limited number of adult mice possible for us to study has prevented more detailed investigations including, eg, feeding experiments with cholesterol. From the observations made thus far on the unique mice generated in 2003 some important conclusions can be drawn, however. Because of the limited number of mice available, and to avoid problems with different genetic backgrounds, we have only compared Dhcr24−/− mice with Dhcr24+/− mice in the present work. In a limited number of early preliminary experiments (not shown) we showed that the Dhcr24+/− mice are identical to the Dhcr24−/− mice with respect to content of desmosterol and expression of various genes.

Methods

The detailed methodology is available as a supplement (http://atvb.ahajournals.org).

Animals

Male mice with a Dhcr24 knockout were generated and maintained as described previously. In most studies the mice had an age of 12 weeks. For further details, see the supplemental materials.

Lipoprotein and Lipid Pattern

Lipoproteins were separated with an automated gel filtration system. For details, see the supplemental materials.

Sterol Analysis in Plasma and Liver

Levels of cholesterol, desmosterol, and oxysterols in circulation, plasma, and liver were determined by isotope dilution–mass spectrometry and use of deuterium-labeled internal standards. For details, see the supplemental materials.

Analyses of Bile Acids

Bile acids in bile were identified after hydrolysis by combined gas chromatography–mass spectrometry and quantitated either by gas chromatography or by isotope dilution–mass spectrometry. Unhydrolysed bile acids were analyzed by electrospray-mass spectrometry. For details, see the supplemental materials.

Analyses of Bile Acids and Sterols in Feces

Fecal sterols and bile acids were identified by combined gas chromatography–mass spectrometry and quantitated by gas chromatography. For details, see the supplemental materials.

Real-Time Polymerase Chain Reaction Analyses

The relative amount of target mRNA was quantitated with use of TaqMan or SYBR Green systems and the specificity of all the polymerase chain reaction (PCR) products was confirmed by sequencing. For details, see the supplemental materials.

Statistics

With the exception of the results shown in Figure 4 data are presented as mean±SEM. With one exception (3-hydroxy-3-methylglutaryl [HMG] coenzyme A [CoA] reductase activity) the normal distribution allowed use of Student t test.

Results

Lipoprotein and Lipid Pattern in the Circulation of the Dhcr24−/− Mice

In accordance with the previous work, the plasma levels of 3β-hydroxy-5-unsaturated steroids (99% cholesterol in the case of Dhcr24±/− mice, about 97% desmosterol in the case of Dhcr24−/− mice) were decreased by almost 50% in the Dhcr24−/− mice as compared with the Dhcr24+/− mice. In contrast, the levels of triglycerides were about double those of the Dhcr24+/− mice.

Figure 1 shows the separation of plasma lipoproteins by size exclusion chromatography and online determination of their lipid components in the 2 groups of animals. Most of the increased triglycerides in plasma of the Dhcr24−/− mice was present in the VLDL fraction. Also total 3β-hydroxy-5-unsaturated steroids and phospholipids were increased in this fraction. The HDL and LDL fractions of the Dhcr24−/− mice had reduced content of all 3 lipid classes.

Fecal Excretion of Neutral Steroids

The fecal excretion of plant sterols was similar in the 2 different groups of animals (Figure 2A and supplemental Table II). If taking into account that the knockout mice had a weight about 25% lower than the heterozygotes and compensating for differences in body weight, the excretion of plant sterols was about 20% higher from the knockouts.

There was a marked and highly significant (P<0.001) increase in the excretion of C27 neutral steroids (about 4-fold) from the knockout mice as compared with the heterozygotes (Figure 2B and Supplemental Table III). If compensating for the lower body weight of the knockout mice, the difference between the 2 groups was even larger. The fecal excretion from the knockout mice was mainly desmosterol (about 97%), whereas the excretion from the heterozygotes mainly consisted of cholesterol (91%).

Composition of Bile Acids in Bile and Feces and Amounts of Different Bile Acids Excreted in Feces

Using combined gas chromatography–mass spectrometry, normal bile acids were shown to be present in a hydrolyzed sample of bile from a knockout mouse. The same bile acids were found in a bile sample from the wild-type mouse and from a heterozygote. As shown in Figure 3A, the relative proportions of the bile acids were different in a sample from a heterozygote and a knockout mouse. In the bile from the heterozygote, cholic acid was dominating (54%) followed by β-muricholic acid (32%) and α-muricholic acid (8%). In the bile sample from the knockout mouse β-muricholic acid was
dominating (53%) followed by cholic acid (19%) and \( ^7 \)-muricholic acid (7%). The alkaline hydrolysis used in the above analysis does not give cleave sulfate esters and does not give information about the type of conjugate present in bile. To obtain qualitative information about this, samples of unhydrolysed bile were analyzed by electrospray mass spectrometry. In similarity with a previous report on electrospray mass spectrometry of mouse bile,10 the major peak appearing both in the analysis of bile from the heterozygote and that from the knockout mouse had a \( m/z \) 514, corresponding to taurine conjugate of trihydroxy-bile acid (cholic acid or \( ^7 \)-muricholic acids). A small peak appeared with \( m/z \) 498, corresponding to taurine conjugate of dihydroxy bile acid (chenodeoxycholic acid and/or deoxycholic acid). No significant peaks corresponding to sulfated bile acids were seen.

Figure 3B summarizes the results of quantitative measurements of fecal excretion of all the different bile acids in heterozygotes (n=6) and gene knockout males (n=6). The total excretion of bile acids was not significantly different in the 2 different groups. If taking into account the lower body weight of the knockout animals, the fecal excretion of bile acids was however about 25% higher in these animals. Excretion of cholic acid was significantly lower in the knockout mice than in the heterozygotes (\( P < 0.01 \)). There was also a tendency toward higher excretion of muricholic acids in the knockout mice.

Relation Between Bile Acids and Neutral Sterols in Bile
The very high fecal excretion of neutral sterols may in part be attributable to an increased biliary excretion, and thus it was considered important to measure the molar ratio between neutral sterols and bile acids in bile. In bile from 4 heterozygotes this ratio was found to be 0.06±0.02, whereas the corresponding ratio was 0.34±0.10 in 4 knockout mice (mean±SEM, \( P < 0.01 \)). There was also a tendency toward higher excretion of muricholic acids in the knockout mice.

Oxysterols in Circulation
In accordance with previous studies,10 7\( \alpha \)-hydroxycholesterol, 24-hydroxycholesterol and 27-hydroxycholesterol were the major oxysterols in the circulation of the heterozygotes.
The levels of these oxysterols were reduced by 72% to 93% in the knockout mice. In plasma of the knockout mice an oxysterol was present that had a polarity slightly higher than that of 27-hydroxycholesterol. This oxysterol had a mass spectrum identical with that of 27-hydroxylated desmosterol produced by action of sterol 27-hydroxylase on desmosterol (supplemental Figure V). The level of 27-hydroxydesmosterol was about 7 times higher than the level of 27-hydroxycholesterol in the heterozygote. The level of 27-hydroxydesmosterol was found to be present in about the same high concentration also in the circulation of a female Dhcr24+/H11002/H11002 mouse. Attempts to find 7α-hydroxylated desmosterol in plasma failed both in male and female Dhcr24+/H11002/H11002 mice.

Measurements of Plant Sterols in Plasma and Liver

Levels of plant sterols in plasma and liver may be used as a surrogate marker for absorption of neutral steroids. As shown in Table 2 there was a marked and highly significant reduction in the levels of the 2 plant sterols in both compartments. This reduction was not caused by a reduced intake of plant sterols as evident from the similar fecal excretion of plant sterols from heterozygous and knock out mice (Figure 2).

Measurements of Hepatic mRNA Levels

Figure 4 shows hepatic mRNA levels measured by real-time PCR in 4 male knockout mice (age 6 to 12 weeks) and 6 heterozygous mice (age 5 to 12 weeks). There was a statistically significant upregulation of Srebp-1c, Srebp-2, HMG-CoA reductase, HMG-CoA synthase, Cyp7a1, Abcg5, Abcg8, and fatty acid synthase. There was a slight reduction of the mRNA levels corresponding to Cyp27 and a very marked reduction in the mRNA levels of cyp7b1. The slight changes in the mRNA levels of the LDL receptor and squalene epoxidase were not statistically significant. Small heterodimer partner (Shp) as well as Squalene epoxidase, Cyp8b1, and Abca1 were not affected by the gene disruption.

Similar results as above were obtained also in microarray analyses (results not shown).

Western Blotting of ABCG5

The ratio between Abcg5 and β-Actin protein was 1.5±0.3 in the analysis of membranes from liver of 3 knockout animals. The corresponding figure for 6 heterozygotes was 0.6±0.1 (mean±SEM, P=0.01). For the Western blottings, See supplemental Figure VI.

Assay of HMG CoA Reductase Activity

HMG CoA reductase activity was measured in liver microsomes from a group of knockout animals about 12 weeks of age (n=4) and a group of heterozygotes of the same age.
The activity was almost 5-fold higher in the knockout mice (n=4) than in the heterozygotes (1.50.5 versus 0.30.16 nmol/min/mg, P=0.02 after log. transformation).

Testing Whether Desmosterol Is a Substrate for CYP27A1
CYP27A1 was incubated with desmosterol as substrate together with adrenodoxin reductase and adrenodoxin as described previously. In a parallel experiment the same amount of cholesterol was used as substrate. In a typical set of experiments about 5% of the cholesterol was converted into 27-hydroxycholesterol. In the parallel experiment with desmosterol about 13% of this steroid was converted into a product with a mass spectrum identical to that obtained in connection with analysis of a serum sample from a Dhcr24-/- mouse (see supplemental Figure V).

Testing Whether Desmosterol Is a Substrate for CYP7A1
CYP7A1 was incubated together with NADPH and NADPH reductase under the same conditions as previously described. In a parallel experiment cholesterol was used as substrate. In a typical set of experiment the conversion of cholesterol into 7α-hydroxycholesterol was about 6%. The formation of 7α-hydroxydesmosterol in the parallel experiment was about 5%.

Testing Whether There Is a Formation of Cholesterol 24,25-Epoxide From Desmosterol
Chloroform extraction of livers of Dhcr24-/- as well as livers of wild-type mice with subsequent work-up, derivatization, and analysis by combined gas chromatography-mass spectrometry failed to result in detection of the epoxide. As judged from parallel analysis of the standard compound, levels of the epoxide >10 ng/g would have been detected under the conditions used.

Discussion
It is evident from the present animal model that desmosterol can replace cholesterol in many but not all of its functions. It has been shown previously that desmosterol can substitute for cholesterol in cultured fibroblasts without deleterious effects. Furthermore most cholesterol in the developing and early neonatal retina in rats can be replaced with desmosterol, both structurally and functionally.

Surprisingly, the -/- mice produced normal bile acids in about normal amounts. It was shown that desmosterol can serve as a substrate both in the classical bile acid biosynthesis pathway starting with a 7α-hydroxylation and in the alternative pathway starting with a 27-hydroxylation. Interestingly, the gene coding for the rate-limiting enzyme in the classical pathway, Cyp7a1, was upregulated. Part of the explanation for this may be the lower production of cholic acid, the major suppressor of Cyp7a1 in mice. A contributing factor may be that Cyp7a1 is regulated by a LXR-dependent mechanism.

The gene coding for the rate-limiting enzyme in the alternative pathway, Cyp27a1, was slightly downregulated by the gene disruption (Figure 4). That this enzyme is active on desmosterol in the alternative pathway was evident both from our in vitro experiment and from the finding that there were high levels of 27-hydroxylated desmosterol in the circulation.
The fact that we did not find any 7α-hydroxylated desmosterol in the circulation, but high levels of 27-hydroxylated desmosterol, is consistent with the possibility that a substantial part of bile acid synthesis in the Dhcr24-deficient mice may occur by a primary 27-hydroxylation of desmosterol rather than by a primary 7α-hydroxylation. In our in vitro experiments with sterol 27-hydroxylase, desmosterol appeared to be a more efficient substrate than cholesterol. This is in line with the previous demonstration that the CYP27 activity seems to increase with the polarity of the substrate.11 Because the transport of cholesterol into the inner mitochondrial membranes is a limiting factor for overall sterol 27-hydroxylase activity,16 and because desmosterol is able to pass lipophilic membranes at a considerably higher rate than cholesterol, this may also be of importance for a high activity of the sterol 27-hydroxylase. A conversion of cholesterol into bile acids by the alternative pathway initiated by a 27-hydroxylation of cholesterol involves participation of the oxysterol 7α-hydroxylase Cyp7b1. The mRNA level of this enzyme was downregulated in the Dhcr24−/− mice. This finding is in accordance with the recent report by Uppal et al that activation of LXRs markedly suppresses expression of Cyp7b1.17 In view of the very high capacity of the oxysterol 7α-hydroxylase to hydroxylate side-chain oxidized oxysterols,18 the downregulation of the gene may not be critical for the overall conversion of cholesterol into bile acids by the alternative pathway.

The alternative pathway for formation of bile acids from cholesterol is known to give chenodeoxycholic acid and its metabolites as major products.19 The increased content of muricholic acids and the reduced content of cholic acid in the bile of the Dhcr24−/− mice is consistent with this.

It is noteworthy that expression of Srebp-1c was higher than that of Srebp-2, probably reflecting the fact that Srebp-1c in contrast to Srebp-2 is one of the genes activated by the LXR-mechanism. Both genes may be upregulated as a consequence of sterol depletion. It is known that Srebp-2 preferentially activates genes of cholesterol metabolism whereas Srebp-1c preferentially activates genes of fatty acid and triglyceride metabolism.20 In this connection it is noteworthy that the gene coding for fatty acid synthase was markedly upregulated in the Dhcr24−/− mice. This effect may be part of the explanation for the increased plasma levels of triglyceride-rich VLDL particles in the Dhcr24−/− mice. It was shown in the recent in vitro study by Yang et al that desmosterol inhibits the Srebp-2 pathway. If such an inhibition is of importance under the present in vivo conditions, it is evident that this inhibition is not able to overcome the stimulatory effect of the sterol depletion.

The very marked effect of the gene disruption on the biliary and fecal excretion of neutral sterols is of particular interest in relation to the recent demonstration that desmosterol activates the nuclear receptor LXR.6 Such an activation would be expected to stimulate the LXR-targeted genes Abcg5 and Abcg8 with reduced intestinal absorption and increased biliary excretion of cholesterol and plant sterols as a consequence. It has been reported that treatment of mice with the LXR agonist GW3965 results in 74% increase in biliary secretion of cholesterol and a 2-fold increase in fecal neutral sterol excretion.21 The even higher fecal excretion of neutral sterols found in our animal model may be attributable to the chronic state with a combination of reduced absorption and increased synthesis of neutral sterols.

It should be noted that although the pattern of expression of the above genes are in accordance with a general stimulation of the LXR-target genes, the changes were relatively modest in relation to the very marked effect observed on fecal excretion of neutral sterols. Surprisingly, the gene coding for the cholesterol transporter Abca1, which is also stimulated by LXR, was not increased. It seems possible that replacing cholesterol with desmosterol in the membranes may modify the rate of transcription or the stability of the mRNA species or both.

The changed distribution of lipids in the different lipoproteins in the circulation (Figure 1) are likely to reflect a mechanism compensating for the reduced levels of 3β-hydroxy-5-unsaturated steroids in the Dhcr24−/− mice. The VLDL-fraction of these mice contained twice as much sterol as the corresponding fraction from Dhcr24+/+ mice, whereas the situation was reversed for HDL. In theory, a LXR-dependent upregulation of the fatty acid synthase may be a driving force for a higher synthesis of triglycerides and increased secretion of VLDL.

Because epoxidation of desmosterol gives cholesterol 24,25-epoxide, which is a very efficient ligand and activator of LXR,22 we tested the hypothesis that this compound is formed from desmosterol in the Dhcr24−/− mice. In spite of extensive efforts, we failed to demonstrate detectable levels of the epoxide in the plasma and liver of the knockout animals. Thus it seems likely that it is desmosterol itself rather than a metabolite that is the direct activator of the LXR gene.

It is interesting to compare the present animal model with the mouse model for the Smith-Lemli-Opitz syndrome, with a mutation in the Dhcr7 gene, causing accumulation of 7-dehydrocholesterol.23,24 These very short-living mice also have markedly reduced tissue levels of cholesterol and total sterols. Despite this, the mRNA levels for HMG-CoA reductase, the LDL receptor and Srebp-2 are essentially normal. In contrast to mRNA, protein levels and activities of HMG-CoA reductase are markedly reduced and it was shown that 7-dehydrocholesterol accelerates proteolysis of HMG-CoA reductase.23 In another study neither 7-dehydrocholesterol nor desmosterol affected reductase ubiquitination and degradation in SV-589 cells.25

To summarize, we have shown that cholesterol homeostasis in Dhc24 knockout mice is characterized by strong activation of LXR-targeted genes and by mechanisms compensating for cholesterol depletion. The very high fecal excretion of neutral sterols, most probably as a consequence of the LXR activation and upregulation of Abcg5/8 may explain why the Dhcr24 knockout mice are unable to accumulate dietary cholesterol. The study also illustrates the importance of the integrity of the structure of cholesterol molecule. Introduction of a single double bond in the steroid side chain is thus compatible with life although disturbances appear in the steroid homeostasis. In contrast a double bond in the steroid nucleus gives more serious consequences.24,25
Sources of Funding
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Disclosures
A.B., M.S., and E.F. are employed by Quark Biotech Inc, the firm that initially developed the present animal model.

References


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Supplementary materials

Methods

Steroids

Labelled and unlabelled steroids were the same as those used in our previous work (1,7,11,12,13,18).

Animals

Male mice with a Dhcr24 knockout were generated and maintained as described in detail in the preceding paper (1). Following the suckling period, the animals were provided with a commercial rodent diet (Harlan Teklad Rat/Mouse Diet) ad libitum. In most studies the mice had an age of 12 weeks, but in one study (Fig. 4) there was a range between 5 and 12 weeks.

Analysis of lipoprotein patterns and their content of cholesterol, triglycerides and phospholipids

Lipoproteins were separated essentially as previously described (10) using a Superose®-& PC 3.2/30 column (Pharmacia Biothech, Uppsala, Sweden).

Sterol analysis in plasma and liver

Levels of cholesterol, desmosterol and oxysterols in circulation, plasma and liver were determined by isotope dilution – mass spectrometry as described previously (1,12,13). 27-Hydroxydesmosterol was identified (cf results) by mass spectrometry as trimethylsilyl ether and quantitated with use of deuterium labelled 27-hydroxycholesterol as internal standard. In this assay the total ion current instead of a specific ion was used and the ratio between the area of the peak corresponding to the internal standard and the area of the peak corresponding to 27-hydroxydesmosterol was used for the quantitation.

Analyses of bile acids in bile

Bile acids were analysed by gas chromatography under the conditions described previously (12,13) The samples were analysed by repetitive scanning for identification of all bile acids present in the samples. In addition, cholic acid, chenodeoxycholic acid and deoxycholic acid were quantitated by isotope dilution-mass spectrometry with the use of deuterium labeled internal standards (12,13). The other bile acids were quantitated from the chromatogram (total ion current) obtained in analysis of material to which no internal standard had been added (12). Unhydrolysed bile acids were analysed by electrospray mass spectrometry using a Quattro micro triple quadrupole mass spectrometer. The general conditions for pre-treatment and analysis of the samples were as described previously (12).
Analysis of bile acids and sterols in faeces

Faeces were collected from 4 individual mice belonging to the two different groups as shown in Table 1 and Fig. 2. The general methodology used was that described by Grundy, Ahrens and Miettinen (14) for the analysis of faecal bile acids and the method by Miettinen (15) for faecal neutral sterols.

Real-Time PCR analyses

The relative amount of target mRNA was quantified by singleplex Real Time RT-PCR analysis on an ABI PRISM® 7000 Sequence Detection System, using m-Hprt (TaqMan or SYBR Green) as an endogenous control in parallel assays. The primers used are presented in Table 1. The specificity of all the PCR products was confirmed by sequencing. In all SYBR Green assays a final concentration of 200 nM or primers were used. For all SYBR Green assays a melting curve was obtained for each PCR product after each run to confirm that the signal corresponded to a unique amplification.

Relative mRNA levels were calculated according to the comparative threshold cycle method (according to User Bulletin #2: ABI PRISM 7700 Sequence Detection System).

Western Blotting of liver membranes

Antibodies towards ABCG5 was a generous gift by Dr Liqing Yu, Dept of Pathology, Section of Lipid Sciences, Wake Forest University School of Medicine, Winston-Salem, USA.

Assay of HMG CoA reductase activity in liver microsomes

Preparation of liver microsomes and assay of HMG CoA reductase was performed as described previously (16) using a modification of the method described by Brown and Goldstein (17).

Incubations with CYP7A1 and CYP27A1

Recombinant CYP27A1 (18), adrenodoxin, adrenodoxin reductase and NADPH were incubated under the conditions described previously (18) together with the substrate (20 µg cholesterol or 20 µg desmosterol). Recombinant CYP7A1 (19) together with NADPH cytochrome P450 reductase and NADPH were incubated under the conditions described previously (19) together with the substrate (20µg cholesterol or desmosterol). The extracted product was analyzed by combined gas chromatography-mass spectrometry as described previously (18,20).
Statistics

With one exception (Fig. 4) all the results are presented as mean +/- SEM in the tables and figures. With one exception the normal distribution allowed us to use Student’s t-test directly. In one case (HMG CoA reductase) this test had to be used after a log-transformation. For clarity of presentation, the Y-axis in Fig. 4 is depicted with a log scale to allow a better evaluation of both increase and decrease of the mRNA levels. In view of the fact that the calculation of the mRNA levels has to include the variance of both the target gene and the house-keeping gene we present the variations of the mRNA levels shown in Fig. 4 in the form of a range rather than SEM.
Table 1. Sequences of Taq-Man primers and probes and SYBR Green primers used for quantitations by real time PCR

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<td>Srebp1c</td>
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<td>5´-ATGTGACAGAGGAGG-3´</td>
</tr>
<tr>
<td>Srebp2</td>
<td>5´-GGGTGTGCTGCTGCTTCTG-3´ 5´-GTCCTGACATGAGGAGG-3´</td>
<td>5´-ATGTGACAGAGGAGG-3´</td>
</tr>
<tr>
<td>Sqle</td>
<td>5´-GACCAAGTCTGCTGCTTCTG-3´ 5´-GTCCTGACATGAGGAGG-3´</td>
<td>5´-ATGTGACAGAGGAGG-3´</td>
</tr>
<tr>
<td>Hprt</td>
<td>5´-GGTGAAGTTTCTGCTGCTTCTG-3´ 5´-GTCCTGACATGAGGAGG-3´</td>
<td>5´-ATGTGACAGAGGAGG-3´</td>
</tr>
</tbody>
</table>
Table 2. Faecal excretion of plant sterols from Dhcr24+/- and Dhcr24-/- mice

<table>
<thead>
<tr>
<th>Plant sterol</th>
<th>Dhcr24+/- (µg/24h)</th>
<th>Dhcr24-/- (µg/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl coprostanol</td>
<td>52±20</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Ethylcoprostanol, Campesterol</td>
<td>681±75 257±23</td>
<td>473±38 198±11</td>
</tr>
<tr>
<td>Ethylcoprostanone</td>
<td>1638±119 623±51</td>
<td>1350±85 514±33</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>3526±284</td>
<td>2860±165</td>
</tr>
<tr>
<td>Sitostanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total plant sterols</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurement in 6 heterozygots and 6 knockouts 3 months of age. The figures given are mean ±SEM.

Mixture of ethylcoprostanol and campesterol that was quantitated together.

The quantitation includes two plant sterols (C28 and C29) that were never identified and present in minor amounts.

Table 3. Faecal excretion of C27 neutral sterols from Dhcr42+/- and Dhcr24-/- mice

<table>
<thead>
<tr>
<th>Neutral sterol</th>
<th>Dhcr42+/- (µg/24h)</th>
<th>Dhcr24-/- (µg/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprostanol</td>
<td>72±17</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Cholesterol+ cholestanol</td>
<td>692±48</td>
<td>91±24</td>
</tr>
<tr>
<td>Desmosterol</td>
<td>&lt;5</td>
<td>3740±274</td>
</tr>
</tbody>
</table>

Measurement in 6 heterozygotes and 6 knockouts 3 months of age. Figures are given as mean±SEM.
Legends to Figures

Fig. 5. Mass spectrum of trimethylsilyl derivative of 27-hydroxydesmosterol present in plasma of a male Dhcr24-/- mouse (A). An identical spectrum was obtained after incubation of desmosterol with CYP27 (B). Characteristic fragments are seen at m/z 544 (M), m/z 454 (M-90). The fragments at m/z 373 (loss of steroid side-chain), m/z 371, m/z 253 m/z 213 and m/z 129 are seen also in the mass spectrum of trimethylsilyl ether of desmosterol.

Fig. 6. Western blotting of membrane preparations from liver of one Dhcr24-/-, two +/- and one +/+ mouse using antibodies towards Abcg5 and Actin.