RESEARCH COMMUNICATION

Sterol carrier protein 2 participates in hypersecretion of biliary cholesterol during gallstone formation in genetically gallstone-susceptible mice

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In inbred mice, susceptibility to cholesterol gallstone disease is conferred by *Lith* genes, which in part promote hypersecretion of cholesterol into bile in response to a high-fat/cholesterol/cholic acid (lithogenic) diet. Because cytosolic sterol carrier protein 2 (SCP2) is believed to participate in cellular cholesterol trafficking and is elevated in the liver cytosol of cholesterol gallstone patients, we defined the hepatic expression of SCP2 during cholesterol gallstone formation in gallstone-susceptible C57L and gallstone-resistant AKR mice fed the lithogenic diet. Steady-state cytosolic SCP2 levels in C57L, but not AKR mice increased as a function of time and were correlated positively with biliary cholesterol hypersecretion, cholesterol saturation indices of gall-bladder biles and the appearance of liquid and solid cholesterol crystals leading to gallstone formation. Steady-state mRNA levels increased co-ordinately, consistent with regulation of SCP2 expression at the transcriptional level. Our results suggest that overexpression of SCP2 contributes to biliary cholesterol hypersecretion and the pathogenesis of gallstones in genetically susceptible mice. Because of the different chromosomal localizations of the *Lith* and *Scp2* genes, we postulate that *Lith* genes control SCP2 expression indirectly.

INTRODUCTION

Epidemiological data are consistent with a genetic basis for cholesterol cholelithiasis in some humans [1]. In certain inbred mice strains, a high-fat/cholesterol/cholic acid (lithogenic) diet induces cholesterol gallstones, whereas other strains are resistant [2–4]. More recently, quantitative trait locus analysis has shown that gallstone susceptibility in C57L mice is conferred by at least two *Lith* genes [4,5]. The phenotypes of mice with susceptible *Lith* alleles include hypersecretion of biliary cholesterol, cholesterol supersaturation in gall-bladder biles, rapid cholesterol crystallization and a high prevalence of gallstones [6,7]. We have now demonstrated that the sister gene of P-glycoprotein (*Spgp*), an ATP-dependent canalicular bile salt transporter [8], is over-expressed in gallstone-susceptible mice and co-localizes with *Lith1* on murine chromosome 2 [9].

The molecular mechanisms responsible for the biliary hyper-secretion of cholesterol are not understood. However, based on the well-described inter-relationships between the secretion rates of biliary lipids in humans and laboratory animals [10], elevated bile salt secretion rates that result from overexpression of *Spgp* in gallstone-susceptible C57L mice could not alone account for the observed hypersecretion of biliary cholesterol. Recent evidence [11,12] suggests the involvement of sterol carrier protein 2 (SCP2), a 13 kDa soluble lipid-transfer protein [13], in the transcellular transport of biliary cholesterol. In particular, the isolated observation that SCP2 concentrations were elevated in liver biopsy specimens from cholesterol gallstone patients [12] led us to explore the hypothesis that overexpression of SCP2 facilitates the hypersecretion of biliary cholesterol in response to elevated bile salt secretion rates in gallstone-susceptible inbred mice. Our results demonstrate that overexpression of SCP2 is correlated with biliary cholesterol hypersecretion, and may represent a critical step in the phenotypic expression of gallstone genes.

EXPERIMENTAL

Animals

Male AKR/J and C57L/J mice (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) had free access to water and standard Purina chow (Rodent Diet 5001, St. Louis, MO, U.S.A.) containing a trace ($< 0.02\%$) of cholesterol. All animals were housed in temperature- and light-controlled (light from 06:00 to 18:00 h) rooms, and were allowed to adjust to this environment for at least 2 weeks prior to experiment at 8–10 weeks of age. Experimental protocols, including surgery and euthanasia, were approved by Institutional Animal Care and Use Committees.

Study design

Animals of both strains were fed a lithogenic diet consisting of 15% (w/v) dairy fat, 1% (w/v) cholesterol and 0.5% (w/v) cholic acid [4] for up to 28 days. At 7-day intervals, groups of four or five mice from each strain were anaesthetized at 09:00 h by an intraperitoneal injection of pentobarbital (35 mg/kg body weight) and, at laparotomy, the bile ducts were identified and the cystic duct was ligated. Following cholecystectomy, gall-bladders were opened immediately for microscopic analysis. An acute bile fistula was created by cannulation of the common bile duct with a PE-10 polyethylene catheter (Becton Dickinson, Sparks, MD, U.S.A.). Hepatic biles were collected from sedated animals for 1 h and stored at $-20\,°C$ until analysis. During bile collection, body temperature was monitored using a rectal thermometer, and was kept at $37 \pm 0.5\,°C$ using a heating lamp. At 7-day intervals, livers from separate groups of four or five mice from

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each strain were harvested, snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}\mathrm{C}$ until used for mRNA and protein determinations.

Bile analysis

Lipid analyses were performed according to standard methods, and cholesterol saturation indices of hepatic and gall-bladder biles were calculated as described previously [6,14]. Rates of bile flow were determined gravimetrically, assuming a density of 1 g/ml, which allowed calculation of lipid secretion rates. Polarizing light microscopy (Carl Zeiss Inc., Thornwood, NY, U.S.A.) of fresh gall-bladder biles was performed to confirm the typical sequence of cholesterol pre-crystal and crystal forms that results in the formation of cholesterol gallstones [6].

Western blot analysis

Livers were homogenized, and cytosol was prepared by serial centrifugation [15]. Protein concentrations were assayed as described previously [16] using a protein assay kit from Bio-Rad Laboratories (Hercules, CA, U.S.A.), with BSA as a standard. Preparations of mouse liver cytosol (100 μg) were electrophoresed at room temperature on SDS/15% (w/v)-polyacrylamide gels [17]. Proteins were transferred electrophoretically on to nitrocellulose membranes (0.45 μm pore size), and antigen detection was carried out using a 1:1000 dilution of an affinity-purified rabbit anti-(mouse SCP2) polyclonal antibody (kindly provided by Dr. Udo Seedorf, Institut für Arterioskleroseforschung, Münster, Germany). Detection of immunoreactive protein was accomplished using a chemiluminescence kit (NEN Research Products, Boston, MA, U.S.A.) employing an anti-rabbit horseradish peroxidase-labelled secondary antibody (Dako Chemicals). Quantitative analysis was performed with a GS-700 Imaging Densitometer (Bio-Rad). In order to assess potential contamination of cytosol with peroxisomal proteins [18,19], blots were stripped and re-probed with a 1:1000 dilution of a sheep anti-(bovine catalase) primary antibody (Biodesign International, Kennebunk, ME, U.S.A.) followed by a 1:10000 dilution of rabbit anti-sheep horseradish peroxidase-labelled secondary antibody (Dako Chemicals). In preliminary experiments, we observed no differences in SCP2 or catalase levels between cytosolic preparations from freshly harvested livers or snap-frozen livers.

Northern blot analysis

Mouse liver RNA was isolated (UltraspecII; Biotecx, Houston, TX, U.S.A.) and 15 μg samples were electrophoresed on 1% (w/v) agarose/2.2 M formaldehyde gels and then transferred to GeneScreen Plus membranes (NEN Research Products). A probe consisting of the coding region of rat liver *Scp2* was prepared by reverse transcription–PCR employing total rat liver RNA [20] followed by radiolabelling with [α-^{32}P]dCTP using a random primer labelling kit (Gibco, Gaithersburg, MD, U.S.A.). After overnight hybridization (43 $^{\circ}$C), membranes were washed at 43 $^{\circ}$C with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)/0.1% SDS for 15 min followed by 0.2 × SSC/0.1% SDS for 20 min, and then at 55 $^{\circ}$C with 0.2 × SSC/0.1% SDS for 15 min. Autoradiography and densitometry was employed to quantify steady-state mRNA levels. To control for variations in lane loading, blots were then stripped and re-probed using a mouse β-actin cDNA (Stratagene) labelled with [α-^{32}P]dCTP.

Statistics

Values are reported as means $\pm$ S.D. for four or five animals from each strain. Data were analysed by the unpaired, two-tailed Student's t-test, and were considered to be significantly different at the $P < 0.05$ level.

RESULTS

Figure 1(A) shows that, during the first 7 days, SCP2 levels increased sharply in C57L mice in response to the lithogenic diet, rising to 160% of the basal value, whereas in AKR mice a modest increase (which did not reach statistical significance) took place. No time-dependent changes in catalase levels were observed for either mouse strain (results not shown). Figure 1(B) illustrates the time sequence for the appearance of liquid crystals, cholesterol crystals and gallstones in gall-bladder biles. Consistent with our earlier observations [6], liquid and solid crystals, including anhydrous and cholesterol monohydrate crystals, formed in gall-bladder biles of C57L mice beginning at day 7, and gallstones were observed at day 28. In contrast, only liquid
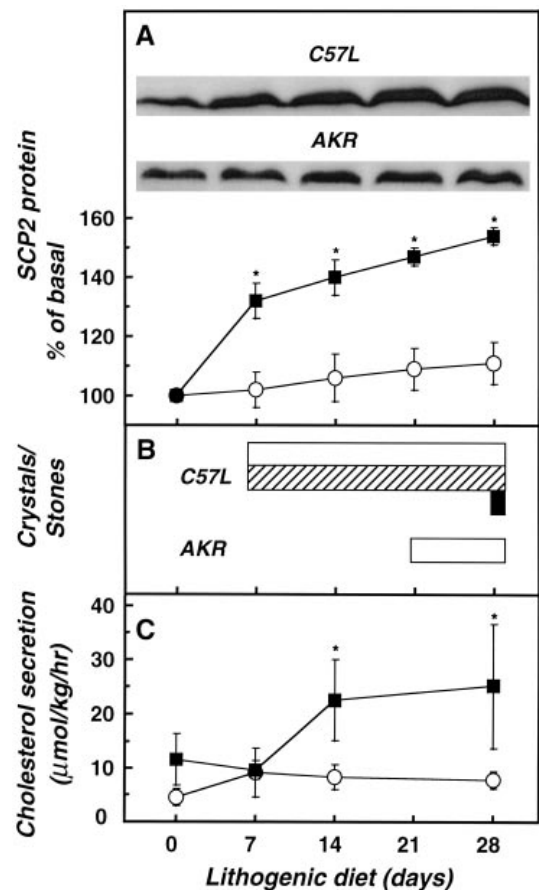
Figure 1   Time course of changes in SCP2 protein levels, cholesterol crystallization and cholesterol secretion rates

Male C57L (■) and AKR (○) mice were fed a lithogenic diet for 28 days. At the time points indicated, gall-bladder and hepatic biles were collected for analysis of the cholesterol crystallization sequences and measurement of biliary lipid secretion rates. Livers were harvested for determination of cytosolic SCP2 protein levels. Cytosolic SCP2 protein levels (**A**) are plotted as a function of time. Each point represents the mean $\pm$ S.D. (*$P < 0.05$ compared with AKR mice). A representative Western blot is also shown in (**A**). Panel (**B**) shows time points at which various phase separations were observed in biles (open bar, liquid crystals; hatched bar, solid crystals; solid bar, cholesterol gallstones). Biliary cholesterol secretion rates are plotted in (**C**).
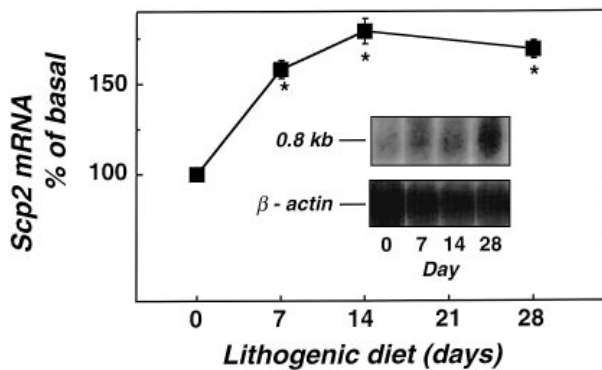
Figure 2   Time course of changes in *Scp2* mRNA levels in C57L mice

Steady-state *Scp2* mRNA levels in male C57L mice are plotted as a function of time. The inset shows a representative Northern blot demonstrating increasing steady-state levels of the 0.8 kb mRNA transcript for *Scp2*, together with β-actin loading controls. Each point represents the mean $\pm$ S.D. ($^*P < 0.05$ compared with basal value).

crystals, but not cholesterol crystals or gallstones, occurred in biles of AKR mice on the lithogenic diet; these appeared after 3 weeks.

Figure 1(C) displays the time course of biliary cholesterol secretion rates for both strains of mice. Basal cholesterol secretion rates (i.e. prior to instituting the lithogenic diet) were 2.5-fold higher in C57L than in AKR mice. Over the 28-day period, cholesterol secretion rates increased only slightly in AKR mice. In C57L mice, biliary cholesterol secretion rates remained constant for the first 7 days and then rose markedly, levelling off after 14 days. As observed for the cholesterol secretion rate, the coupling ratio of biliary cholesterol secretion to bile salt secretion (i.e. mol of cholesterol molecules secreted into bile per mol of secreted bile salts) was higher in the basal state for C57L compared with AKR mice (0.081 compared with 0.045), and each of these values doubled over the 28-day time period. In AKR mice, doubling of the coupling ratio was attributable to a decrement in bile salt secretion rate (results not shown). In contrast, doubling the coupling ratio in C57L mice was principally because the cholesterol secretion rate increased (Figure 1C) and the bile salt secretion rate remained constant (results not shown). The exception was at day 7, at which point the cholesterol secretion rate was unchanged in C57L mice, but there was a transient decrease in the bile salt secretion rate. The net result was a 30 % increase in the coupling ratio of cholesterol secretion to bile salt secretion compared with day 0.

To investigate the expression of the gene encoding SCP2, we determined steady-state *Scp2* mRNA expression during the time course of feeding the lithogenic diet. Of the four distinct mRNA transcripts for *Scp2*, the 0.8 kb transcript has been demonstrated to encode an SCP2 precursor protein that is post-translationally modified to form the mature 13 kDa SCP2 [21]. Figure 2 illustrates the time course of steady-state mRNA levels for the 0.8 kb *Scp2* transcript in C57L mice upon 4 weeks' challenge with the lithogenic diet. The *Scp2* mRNA levels had increased by 1.8-fold ($P < 0.05$) at day 14 after commencing the lithogenic diet, and then levelled off. In contrast, mRNA levels for *Scp2* did not change in AKR mice (results not shown).

DISCUSSION

Several lines of evidence both *in vivo* and *in vitro* indicate that SCP2 participates in intracellular trafficking of cholesterol

[22–26]. These observations, taken together with a report that SCP2 levels are elevated in the hepatic cytosol of cholesterol gallstone patients [12], suggest a possible role for SCP2 in biliary cholesterol secretion. Ongoing research in our laboratories has focused on phenotypic and genotypic analyses of gallstone-susceptible and gallstone-resistant mice strains, with the principal objective of isolating and characterizing the *Lith* genes [4,6,7,27]. These studies support the concept that the primary patho-physiological defect leading to cholesterol gallstone formation in inbred mice is the hypersecretion of biliary cholesterol [7], as is the case in human gallstone subjects [28]. To explore further the contribution of SCP2 to the hepatocellular trafficking of biliary cholesterol, we examined its expression during the stages of cholesterol gallstone formation in a gallstone-susceptible strain (C57L) of mice [6] and compared the response in an inbred strain (AKR) of mice resistant to gallstone formation.

The principal finding was that significant increases in steady-state SCP2 levels occurred in cytosolic fractions prepared from gallstone-susceptible C57L mice, but not in those from resistant AKR mice (Figure 1A). Although it has been suggested that SCP2 may be strictly localized to peroxisomes in most tissues (reviewed in [13]), an appreciable proportion of SCP2, at least in liver, is present in the cytosol [18,19,29,30]. An absence of time-dependent changes in soluble catalase levels most probably excluded the possibility that increases in SCP2 reflected contamination of cytosolic preparations with peroxisomal SCP2 [13,18,19]. Co-ordinate increases in steady-state mRNA and cytosolic SCP2 protein levels (Figures 1 and 2) suggest transcriptional up-regulation in *Scp2* gene expression in gallstone-susceptible C57L mice as the mechanism for the observed increase in SCP2 protein (Figure 2). However, because both transcriptional and post-transcriptional regulation of SCP2 have been demonstrated [31], the possibility that increases in steady-state mRNA and cytosolic protein levels reflect decreased rates of degradation has not been excluded.

Consistent with a contributory role for SCP2 in the lithogenic process, elevations in steady-state cytosolic SCP2 concentrations in C57L mice corresponded temporally with the appearance of liquid and solid crystals in gall-bladder biles (Figure 1B). Over the 28-day period of lithogenic diet feeding, biliary cholesterol levels increased significantly in C57L mice and slightly in AKR mice. As observed previously [6,7], bile salt secretion rates in C57L mice decreased transiently after 7 days on the lithogenic diet. However, this decline was not associated with a decline in the cholesterol secretion rate (Figure 1C). Consequently, at all time points the lithogenic diet induced relative biliary hypersecretion of cholesterol in C57L mice, but not in AKR mice.

The cholesterol saturation index is a quantitative measure of the biliary cholesterol content related to the equilibrium micellar solubilizing capacity [14], and Figure 3 correlates steady-state SCP2 levels with cholesterol saturation indices for hepatic biles (the present study) and for gall-bladder biles (previously determined in our laboratories [6]) in gallstone-susceptible C57L mice. In hepatic biles with low micellar solubilizing capacities, our data indicate that cholesterol saturation indices are high prior to the lithogenic diet and do not increase strongly, despite cholesterol hypersecretion induced by SCP2 up-regulation. Because hepatic biles are dilute ($\sim$ 1–2 g/dl total lipid concentration), the region of the appropriate phase diagrams containing the relative lipid compositions does not support nucleation of cholesterol crystals, despite the uniformly high cholesterol saturation indices (Figure 3) [32,33]. In contrast, cytosolic SCP2 concentrations become highly predictive of cholesterol saturation indices in gall-bladder bile. This suggests that up-regulation of SCP2 may be responsible for hepatic transport and biliary
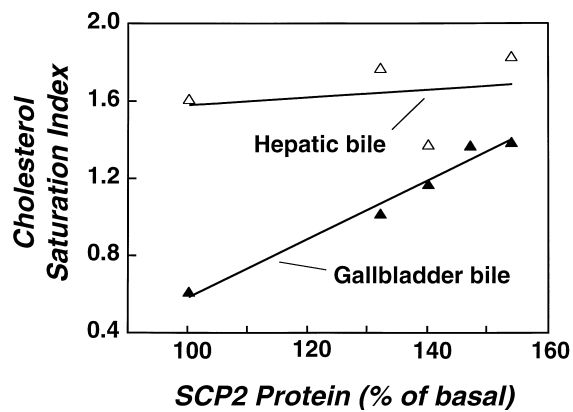
Figure 3   Correlation between biliary cholesterol saturation indices and SCP2 protein in C57L mice

Cholesterol saturation indices for hepatic biles (△) and gall-bladder biles (▲) are plotted as a function of steady-state SCP2 protein levels (see Figure 1) in the hepatic cytosol of male C57L mice during feeding the lithogenic diet for 28 days.

secretion of cholesterol in excess of that which may be solubilized at equilibrium in the gall-bladders of gallstone-susceptible mice [14]. Because AKR mice, but not C57 mice, responded to the lithogenic diet by down-regulating the rate-limiting step in cholesterol biosynthesis [3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase] [4,27], our data suggest the possibility that SCP2 is functioning to transport excess newly synthesized cholesterol into bile. Such an explanation would be consistent with experiments [11] in which antisense oligonucleotides were used to decrease hepatic SCP2 levels, resulting in proportional decreases in the biliary secretion of newly synthesized cholesterol.

Because SCP2 is capable of stimulating the biosynthesis of both cholesterol and bile acids, as well as cholesterol esterification (reviewed in [13]), it is essential to consider the possibility that the principal pathophysiological driving force for SCP2 up-regulation may have been alterations in hepatic cholesterol metabolism rather than biliary secretion. Recently, we [27] examined the activities of the principal enzymes of cholesterol metabolism [HMG-CoA reductase, cholesterol 7α-hydroxylase, sterol 27-hydroxylase and acyl-CoA:cholesterol acyltransferase (ACAT)] in livers of C57L and AKR mice under experimental conditions identical with those used in the present study. The lack of down-regulation of HMG-CoA reductase observed in C57L mice suggests that the cholesterol biosynthetic rate remains constant, thereby eliminating the possibility that SCP2 expression was up-regulated to support enhanced cholesterol synthesis. Because both mice strains exhibited similar increases in ACAT activity and decreases in the activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, changes in cholesterol esterification or bile acid biosynthesis could not account for the observed strain-dependent differences in SCP2 expression. Therefore, by exclusion, these findings collectively support a role for SCP2 in the hepatocellular trafficking of biliary cholesterol, a major hepatic source of which appears to be high-density lipoproteins [34].

Up-regulation of SCP2 in concert with hypersecretion of biliary cholesterol prior to the appearance of cholesterol crystals in bile or gallstones strongly suggests a pathogenetic role for this protein in murine cholelithiasis promoted by *lith* genes. Because the structural *Scp2* gene has been mapped to chromosome 4 [35,36], it is currently excluded as a candidate *Lith* gene because

it does not co-localize with any identified quantitative trait locus for gallstone formation [4]. Rather, we infer that one or more *Lith* gene(s) may regulate the expression of *Scp2*. The recent report of a knockout mouse for the gene encoding both SCP2 and SCP$_x$ (a peroxisomal thiolase–SCP2 fusion protein) [37] suggests that a mouse containing a selective disruption in the *Scp2* gene may soon be available, and this would permit further study of the potential role of SCP2 in the hepatocellular trafficking of biliary cholesterol and in gallstone formation.

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