Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation

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Klipsic D, Landrock D, Martin GG, McIntosh AL, Landrock KK, Mackie JT, Schroeder F, Kier AB. Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation. Am J Physiol Gastrointest Liver Physiol 309: G387–G399, 2015. First published June 25, 2015; doi:10.1152/ajpgi.00460.2014.—While a high-cholesterol diet induces hepatic steatosis, the role of intracellular sterol carrier protein-2/sterol carrier protein-x (SCP-2/SCP-x) proteins is unknown. We hypothesized that ablating SCP-2/SCP-x [double knockout (DKO)] would impact hepatic lipids (cholesterol and cholesteryl ester), especially in high-cholesterol-fed mice. DKO did not alter food consumption, and body weight (BW) gain decreased especially in females, concomitant with hepatic steatosis in females and less so in males. DKO-induced steatosis in control-fed wild-type (WT) mice was associated with 1) loss of SCP-2; 2) upregulation of liver fatty acid binding protein (L-FABP); 3) increased mRNA and/or protein levels of sterol regulatory element binding proteins (SREBP1 and SREBP2) as well as increased expression of target genes of cholesterol synthesis (Hmgcs1 and Hmgcr) and fatty acid synthesis (Acc1 and Fas); and 4) cholesteryl ester accumulation was also associated with increased acyl-CoA cholesterol acyltransferase-2 (ACAT2) in males. DKO exacerbated the high-cholesterol diet-induced hepatic cholesterol and glyceride accumulation, without further increasing SREBP1, SREBP2, or target genes. This exacerbation was associated both with loss of SCP-2 and concomitant downregulation of Ceb/Hsl, apolipoprotein B (ApoB), MTP, and/or L-FABP protein expression. DKO diminished the ability to secrete excess cholesterol into bile and oxidize cholesterol to bile acid for biliary excretion, especially in females. This suggested that SCP-2/SCP-x affects cholesterol transport to particular intracellular compartments, with ablation resulting in less to the endoplasmic reticulum for SREBP regulation, making more available for cholesteryl ester synthesis, for cholesteryl-ester storage in lipid droplets, and for bile salt synthesis and/or secretion. These alterations are significant findings, since they affect key processes in regulation of sterol metabolism.

Studies in vitro and with cultured cells show that the 13-kDa sterol carrier protein-2 (SCP-2) binds cholesterol (15, 44, 61, 68, 85, 87, 96, 110), markedly enhancing cholesterol transfer from plasma membranes and lysosomes to plasma membranes, ER, and mitochondria (3, 26, 27, 29, 30, 32, 33, 43, 45, 84, 98, 109). SCP-2 overexpression increases cholesterol uptake as well as transfer from plasma membranes to ER for esterification and cholesteryl ester accumulation in L-cell fibroblasts (11, 67, 95). SCP-2 is an intracellular binding partner with caveolin-1 and SR-B1 and preferentially enhances cholesterol trafficking from cholesterol-rich plasma membrane microdomains in which these receptors are abundantly distributed (3, 8, 88, 97, 98, 115). SCP-2 and sterol carrier protein-x (SCP-x) proteins also contribute to rapid clearance of HDL-cholesterol by facilitating biliary bile acid synthesis (97, 100). By binding and transferring cholesterol to ER, SCP-2 stimulates hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme in hepatic bile acid synthesis (52, 92). Through an alternate transcription site, the SCP-2/SCP-x gene also encodes a larger protein, SCP-x (31, 90), which catalyzes the peroxisomal oxidation of cholesterol’s branched side chain to form bile acids (31, 90). Canalicular secretion of bile acid drives biliary cholesterol secretion (77, 81, 104). These findings suggest a role for SCP-2 in rapid cholesterol intracellular trafficking of HDL-derived cholesterol for biliary excretion, lipoprotein-derived cholesterol to ER for esterification, and potentially regulation of SREBP release.

In vivo support for roles of the SCP-2/SCP-x gene in hepatic cholesterol metabolism and biliary cholesterol secretion comes from studies in both humans and mice. A human SCP-2/SCP-x genetic variation inhibits cholesterol metabolism (23). However, the human studies were performed with only a single patient (23). In mice, SCP-2 overexpression (2, 5, 112), SCP-2 antisense treatment (76), and SCP-2/SCP-x gene ablation (28, 51, 91) significantly impact hepatic cholesterol metabolism and biliary excretion. Despite the fact that hepatic SCP-x expression is severalfold lower in female mice and female humans (6, 9), almost all rodent studies have been conducted with male mice (2, 5, 28, 51, 76, 91, 112). Studies of the impact of SCP-2 in the face of a high-cholesterol diet are even more limited. A single study has examined the effect of SCP-2 overexpression on high-cholesterol-fed mice but only in males (5). The impact of SCP-2/SCP-x gene ablation [double knockout (DKO)] in the context of a high-cholesterol diet is unknown in either sex. Thus the current study examined the impact of the DKO on hepatic cholesterol and biliary phenotype of male and female mice fed a high-cholesterol vs. control diet.
**EXPERIMENTAL PROCEDURES**

**Materials.** Rabbit polyclonal antibodies to the following proteins were obtained as follows: anti-peroxisome proliferator-activated receptor-α (PPARα; PAI-822A) from ThermoFisher Scientific (Rockford, IL), anti-ATP-binding cassette transporter (ABCA1; NA400-105), anti-HMG-CoA synthase (HMGC2; sc-33829), anti-small heterodimer protein (SHP; sc-30169), anti-sterol regulatory element-binding protein 1 (SREBP1; recognizing p68 and p125, sc-367), and anti-sterol regulatory element-binding protein-2 (SREBP2; recognizing mature p68 and ER precursor p125; sc-8151) from Santa Cruz Biotechnology (Santa Cruz, CA) (10); anti-sterol carrier protein 2 (recognizing 58 kDa SCP-x, 15 kDa pro-SCP-2, and 13.2 kDa SCP-2) (10), anti-ACAT2 (ab66259), anti-apoipoprotein B (Apob; ab31992); and anti-cytochrome c oxidase subunit 4 (COX4; ab16056) from Abcam (Cambridge, MA). Goat polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) as follows: anti-cholesterol-7-alpha-hydroxylase (CYP7A1; sc-144246); anti-sterol-27-hydroxylase (CYP27A1; sc-14835); anti-fatty acid transport protein 2 (FATP-2; sc-161311); anti-fatty acid transport protein 4 (FATP-4; sc-5834), anti-apolipoprotein A1 (Apoa1; sc-23606); anti-carnitine palmitoyltransferase 1 (CPT1; sc-31128); anti-phosphatidylinositol transfer protein (PITP; sc-23672); anti-sterol regulatory element-binding protein 2 (SREBP2; sc-8151); anti-microsomal triglyceride transfer protein (MTP; sc-33116); anti-liver-type fatty acid binding protein (L-FABP; sc-16064); anti-liver X receptor (LXR; sc-1201); and anti-farnesoid X receptor (FXR; sc-1205). All reagents and solvents used for each test method were highest grade available.

**Animal care.** Animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Male and female inbred C57BL/6Ncr mice were from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). SCP-2/SCP-x null (DKO) mice on the same C57BL/6Ncr background were generated and backcrossed to C57BL/6NCr to the N6 generation (8). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and β-hydroxybutyrate (β-HB) were quantified utilizing Stanbio diagnostic kits (Boerne, TX).

**Lipid analysis of serum, bile, and liver.** About 0.1-g (wet weight) portions of livers from each mouse were homogenized in 0.5 ml PBS (pH 7.4) with motor-driven pestle (Tekmar, Cincinnati, OH) at 2,000 rpm for 5 min. Liver homogenate and serum protein were determined by a Bradford micro-assay from Bio-Rad Laboratories (cat no. 500-0001; Hercules, CA). Costar 96-well assay plates (Corning, Corning, NY) and a Bio Tek Synergy 2 microplate reader (Bio Tek Instruments, Winoooski, VT) were utilized for liver lipid, serum, and bile assays. Liver homogenate and serum lipids were quantified with Wako diagnostic kits (Richmond, VA) for total cholesterol, free cholesterol, triglyceride, phospholipid, and HDL cholesterol (HDL-C). Liver and serum cholesterol ester concentrations were calculated by subtraction of free cholesterol from total cholesterol. Serum non-HDL-C was calculated by subtracting serum HDL-C from serum total cholesterol. Serum apoA1 and serum apoB and serum, liver, and bile total bile acids were quantified using the Diazyme diagnostic kit (Ponwy, CA). All commercially available diagnostic kits were utilized according to the manufacturer’s instructions, modified for use with 96-well plates and microplate reader as described above.

**In vivo whole body composition.** Mouse total body lean tissue mass (LTM) and fat tissue mass (FTM) were determined in vivo by dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXimus densitometer (Lunar, Madison, WI) as previously described (9). The DEXA instrument was calibrated using a phantom mouse with known bone mineral density and fat tissue mass as described previously (9, 69). DEXA was performed on individual mice at the beginning of the dietary study after mice were fasted overnight (~12 h) and anesthesia-[100 μg/kg ketamine and 10 mg/kg xylazine ip as described previously (9)]. At the end of the study (day 30), DEXA was similarly performed, except mice were euthanized before DEXA. DEXA allowed resolution of bone mass and tissue mass for further resolution into LTM and FTM as described previously (9, 69).

**Serum, liver, and bile collection.** On day 29 of the study, mice were fasted overnight (~12 h) to decrease the influence of recent digestion on serum and liver lipid levels. On day 30, after anesthesia, blood was collected via cardiac puncture, followed by humane euthanasia by cervical dislocation. Blood was stored overnight at 4°C and centrifuged at maximum speed for 20 min, and serum was collected and stored at −80°C. Gall bladders containing bile were separately harvested, and livers were weighed and sectioned. Part of the liver was transferred to a RNA stabilization buffer, RNALater (Ambion, Austin, TX), and stored at −20°C. All remaining tissue samples were flash frozen on dry ice and stored at −80°C for future analysis.

**Liver histopathology and serum markers of liver toxicity.** Liver slices near the porta hepatis were fixed for 24 h in 10% neutral buffered formalin, placed in individual cassettes, stored in 70% alcohol, processed and embedded in paraffin, cut in 4- to 6-μm sections, and hematoxylin and eosin stained for histological evaluation (9, 55). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and β-hydroxybutyrate (β-HB) were quantified utilizing Stanbio diagnostic kits (Boerne, TX).

**Liver cholesterol in SCP-2/SCP-X null mice.** In the cage and improve food consumption accuracy, diets were color-coded (yellow, CO; blue, CH).

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**Hepatic mRNA levels of genes in lipid metabolism.** Total RNA was isolated from liver and purified using the RNeasy mini kit (Qiagen, Valencia, CA) using the manufacturer’s standard protocol. Nucleic acid concentration and quality were determined by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were stored at −80°C. Quantitative real-time PCR (QrtPCR) expression patterns were analyzed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan RNA- to-CT 1-Step PCR Master Mix Reagent kit, gene-specific TaqMan system (Applied Biosystems, Foster City, CA) using TaqMan RNA- to-CT 1-Step PCR Master Mix Reagent kit, gene-specific TaqMan PCR probes and primers, and the following thermal cycler protocol: 48°C for 30 min, 95°C for 10 min, 95°C for 0.15 min, and 60°C for 1.0 min, repeated a total of 60 cycles. TaqMan gene expression assays for specific probes and primers were obtained from Life Technologies.
(Carlsbad, CA) to determine hepatic mRNA levels of ATP-binding cassette subfamily G member 5 (Abcg5; Mm01226965_s1), ATP-binding cassette subfamily G member 8 (Abcg8; Mm00445977_m1), acetyl CoA carboxylase-1 (Accl; Mm01304285_s1), acetyl CoA carboxylase-2 (Acc2; Mm01204657_m1), bile salt export pump (Bsep; Abcl1; Mm00445168_m1), cholesterol ester hydrolase/hormone-sensitive lipase (Cebhs/Hsl; Mm00495359_m1), fatty acid synthase (Fas; Mm00662319_m1), HMC-CoA synthase (Hmgcs1, Mm01304569_ml), HMG-CoA reductase (Hmgcr, Slc10a1; Mm01306289_m1), low-density lipoprotein receptor (Ldlr, Mm01177349_ml), Na+-taurocholate cotransporting polypeptide (Ntcp/ Slco1a1; Mm01302718), organic anion transporting polypeptide 1 (Oatp/Scpl1; Mm01307414_m1), organic anion transporting polypeptide 2 (Oatp2/Slco1b1; Mm00460672_m1), scavenger receptor class B type 1 (Scarb1, Mm00450234_ml), sterol regulatory element binding protein-1 (Srebp1; Mm00550338_m1), and sterol regulatory element binding protein-2 (Srebp2; Mm01306289_m1). Two replicates of each sample reaction (20 µl total volume each) were performed on 96-well optical reaction plates (Applied Biosystems, Foster City, CA). ABI Prism 7000 SDS software (Applied Biosystems) established the threshold cycle from each well. QrtPCR data were normalized to the housekeeping gene 18S RNA for mRNA expression of Abcg5, Abcg8, Acc1, Acc2, Bsep, Cebhs/Hsl, Fas, Hmgcs1, Hmgcr, Ldlr, Ntcp, Oatp1, Oatp2, Scarb1, Srebp1, and Srebp2 made relative to the control mouse group (male WT mice on control diet) for final calculations.

Hepatic levels of proteins in lipid metabolism. SDS-PAGE and Western blot analysis was performed on liver postnuclear supernatants (PNS) as described previously (8) to determine relative protein levels of ACAT2, ABCA1, ApoA1, ApoB, COX4, CPTA1, CYP7A1, CYP27A1, FATP-2, FATP-4, HMGCR, L-FABP, PPARα, PCTP, SCP-2, SCP-x, SREBP-1 (68- and 125-kDa forms), SREBP-2 (68- and 125-kDa forms), FXR, and LXR. Images of the blots were captured using an Epson Perfection V700 Photo scanner (Long Beach, CA). Proteins were quantified by densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD) as described earlier (8). Representative cropped Western blots were inserted into figures and presented similarly as in earlier publications wherein individual blots were separated by a white line/space (46, 56, 74, 99, 100).

Table 1. Effect of SCP-2/SCP-x gene ablation on total food consumption and body weight gain of mice fed a high-cholesterol diet

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<td>CO</td>
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<td>CO</td>
<td>CH</td>
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<tr>
<td>Total food consumption, g</td>
<td>86 ± 3</td>
<td>91 ± 2</td>
<td>86 ± 2</td>
<td>88 ± 2</td>
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<tr>
<td>Body weight gain, g</td>
<td>2.5 ± 0.5</td>
<td>2.7 ± 0.4</td>
<td>2.4 ± 0.6</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>Body weight gain/food consumption, mg/g</td>
<td>30 ± 5</td>
<td>30 ± 3</td>
<td>26 ± 5</td>
<td>18 ± 4</td>
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<tr>
<td>LTM change, g</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.5</td>
<td>0.4 ± 1.2</td>
<td>0.1 ± 0.8</td>
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<tr>
<td>FTM change, g</td>
<td>0.1 ± 0.2</td>
<td>0.5 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>1.4 ± 0.4</td>
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Values represent the mean ± SE, n = 5–7. SCP, sterol carrier protein; WT, wild type; DKO, double knockout; CO, control; CH, cholesterol; LTM, lean tissue mass; FTM, fat tissue mass. *Genotype effect (P < 0.05 for DKO vs. WT within the same diet).

RESULTS

Food intake, whole body phenotype, and liver histology. Neither SCP-2/SCP-x gene ablation (DKO), high-cholesterol diet, nor both altered total food consumption by WT male and female mice (Table 1). Yet, DKO decreased body weight gain and body weight gain/food consumption in female (but not male) mice regardless of diet (Table 1). DEXA showed that, while fat tissue mass was not altered, lean tissue mass was significantly decreased only in high-cholesterol-fed DKO vs. WT mice (Table 1).

Gross analysis of liver did not detect significant alterations due to genotype, high-cholesterol diet, or both. Neither cholesterol diet, DKO, nor both altered liver weight, liver weight/body weight, or liver total protein (Table 2). Histological analysis revealed that the high-cholesterol diet was associated with increased fatty vacuolation of hepatocytes in WT males and females. The DKO did not significantly exacerbate this effect at the histological level (not shown). There were no other significant changes in liver histopathology. Serum AST and ALT values in all groups were <35 and 60 U/l, respectively, well within the normal range of mouse values [blood chemistry and hematology in 8 inbred strains of mice, MPD:Eumorpha. Mouse Phenome Database web site, The Jackson Laboratory, Bar Harbor, ME: http://phenome.jax.org (cited 29 Oct. 2014)]. Thus the altered whole body phenotype or serum and hepatic

Table 2. Effect of SCP-2/SCP-x gene ablation, high-cholesterol diet, and both together on liver parameters

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<td>WT</td>
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<td></td>
<td>CO</td>
<td>CH</td>
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<tr>
<td>Liver weight, g</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>Liver weight/body weight, mg/g</td>
<td>38 ± 2</td>
<td>43 ± 1</td>
<td>40 ± 1</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>Protein, mg/g</td>
<td>130 ± 10</td>
<td>126 ± 12</td>
<td>122 ± 7</td>
<td>130 ± 11</td>
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<tr>
<td>Serum ALT, U/l</td>
<td>54 ± 4</td>
<td>45 ± 4</td>
<td>41 ± 5</td>
<td>40 ± 5</td>
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<tr>
<td>Serum AST, U/l</td>
<td>30 ± 2</td>
<td>33 ± 4</td>
<td>19 ± 2</td>
<td>22 ± 3</td>
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Values represent the means ± SE; n = 6–7. ALT, alanine aminotransferase; AST, aspartate aminotransferase. No significant differences were noted between groups.

Statistical analysis. All result values were stated as the means ± SE. Statistical analysis was performed using one-way ANOVA, followed with the Newman-Keuls multiple comparisons test using either GraphPad software (La Jolla, CA) or SigmaPlot software (Systat, San Jose, CA). Statistical significance was assigned to values with P < 0.05.
changes were not due to toxicity in DKO and high-cholesterol diet groups.

Liver lipids. Both the high-cholesterol diet and DKO impacted hepatic lipid accumulation in a sex-dependent manner. The high-cholesterol diet alone increased hepatic accumulation of neutral lipids the most in WT females, without altering phospholipid levels (Fig. 1, B and C), while also increasing both neutral and phospholipid in WT males (Fig. 1, B and C). With neutral lipids, the cholesterol diet alone increased total cholesterol (free and esterified) and triglycerides in both groups (Fig. 1, D–G). These findings were consistent with earlier studies wherein other mouse strains were fed high-cholesterol diets (53, 58, 59).

DKO alone modestly increased liver total lipid (Fig. 1A), total neutral lipid (Fig. 1B), and phospholipid (Fig. 1C) in control-fed males and even more so in control-fed females. The increases in hepatic neutral lipid were due to increased total cholesterol (Fig. 1D), primarily cholesteryl ester, in both males and females (Fig. 1F). The DKO mice had significantly increased hepatic triacylglyceride only in control-fed females (Fig. 1G). The high-cholesterol diet induced hepatic lipid accumulation, especially in females. Females had the highest hepatic levels of total lipid (Fig. 1A), total neutral lipid (Fig. 1B), total cholesterol (Fig. 1D), free cholesterol (Fig. 1E), cholesteryl ester (Fig. 1F), phospholipid (Fig. 1C), and triacylglyceride (Fig. 1G). These increases were not due to concomitant upregulation of proteins involved in fatty acid uptake (FATP-2 and FATP-4), downregulation of oxidation (CPT1), or altered serum β-hydroxybutyrate levels (not shown).

In summary, the DKO generally exacerbated the cholesterol diet-induced hepatic accumulation of cholesteryl ester and triglyceride, the major neutral lipid species comprising lipid droplets and the core of secreted VLDL. Accumulation of all forms of cholesterol and triglyceride was highest in the DKO females.

Serum lipids. In contrast to the significant lipid accumulation in liver, the high-cholesterol diet, DKO, or both elicited less change in serum lipids in both sexes (Table 3). However, a significant decrease in serum polar/neutral lipid ratio, parallelizing a decrease in this ratio in liver lipids, was detected in response to the high-cholesterol diet in WT males but not females (Table 3). The DKO prevented this decrease in ratio in cholesterol-fed males or females (Table 3). Fractionation of serum neutral lipids also showed little or no impact in high-cholesterol diet, DKO, or both on serum total cholesterol, free cholesterol, cholesteryl ester or triacylglycerol (Fig. 2, A–D). Serum cholesterol distribution in the high-density lipoprotein (HDL-C) fraction (Fig. 3A) and non-HDL fraction (Fig. 3B) again showed little impact of high-cholesterol diet, DKO or both. With the exception of increased ApoB in male control-fed DKO mice (Fig. 3D), there were few changes in ApoA1 and ApoB levels or ApoA1/HDL-C and ApoB/non-HDL-C ratios (Fig. 3, C–F).

In summary, high-cholesterol diet or loss of SCP-x/SCP-x or both together did not or only slightly altered serum levels of lipids and key lipoprotein apolipoproteins.

Hepatic proteins in basolateral uptake and intracellular storage of cholesterol from serum lipoproteins. Liver receptors for HDL and LDL facilitate basolateral cholesterol uptake. However, hepatic accumulation of cholesterol in control-fed male DKO mice was associated only in part with concomitant upregulation of Scarb1, while Ldlr tended to decrease (Fig. 4, A and B). Levels in female control-fed DKO mice as well as in

### Table 3. Effect of SCP-2/SCP-x gene ablation, high-cholesterol diet, and both together on serum lipids

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<tr>
<td>DKO</td>
<td></td>
<td></td>
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<tr>
<td>Total protein, mg/dl</td>
<td>1,260 ± 30</td>
<td>1,140 ± 60</td>
</tr>
<tr>
<td>Total lipid, mg/dl</td>
<td>262 ± 7</td>
<td>222 ± 8†</td>
</tr>
<tr>
<td>Neutral lipid, mg/dl</td>
<td>160 ± 7</td>
<td>143 ± 6</td>
</tr>
<tr>
<td>Phospholipid, mg/dl</td>
<td>99 ± 3</td>
<td>77 ± 4†</td>
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<tr>
<td>Serum polar/neutral lipid, mg/mg</td>
<td>0.65 ± 0.04</td>
<td>0.56 ± 0.03</td>
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<td>Liver polar/neutral lipid, mg/mg</td>
<td>1.5 ± 0.1</td>
<td>1.1 ± 0.1†</td>
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Values represent the mean ± SE; n = 6–7. *Genotype effect (P < 0.05 for DKO vs. WT within the same diet). †Diet effect (P < 0.05 for CO vs. CH diet within the same genotype).
both male and female high-cholesterol-fed DKO mice were not increased (Fig. 4, A and B).

Hepatic storage of cholesterol as cholesteryl ester is regulated by the opposing activities of two key proteins: the synthetic enzyme ACAT2 and the degradative Ceh/Hsl. Hepatic accumulation of cholesterol in control-fed male DKO mice was associated with upregulation of ACAT2, but not Ceh/Hsl, while females were unaltered (Fig. 4, C and D). In contrast, while hepatic cholesterol accumulation in cholesterol-fed male DKO mice was not associated with up- and down-regulation of ACAT2 and Ceh/Hsl, respectively, in females the decreased Ceh/Hsl, concomitant with unaltered ACAT2, appeared to contribute to increased liver cholesterol (Fig. 4, C and D).

In summary, the increased hepatic cholesterol accumulation in DKO mice was not associated with concomitant upregulation of Scarb1 or Ldlr. Accumulation of cholesterol ester in control-fed male, but not female, DKO mice was associated in part with increased expression of ACAT2. The DKO-induced exacerbation of hepatic cholesterol ester accumulation in high-cholesterol-fed mice was associated with decreased Ceh/Hsl rather than increased ACAT2.

Proteins involved in cytosolic transport/targeting of cholesterol. The DKO resulted in complete ablation of both SCP-2 and SCP-x protein expression (Fig. 5, C and D). In control-fed DKO mice, this loss was compensated only in part by concomitant upregulation of the other major bile acid binding/transport protein L-FABP, whose expression was increased significantly in males and trended to increase slightly in females (Fig. 5E). The high-cholesterol diet alone increased expression of L-FABP and SCP-x in WT males but only SCP-2 and SCP-x in WT females (Fig. 5, C–E). DKO prevented the cholesterol diet-induced increase in hepatic L-FABP expression to decrease L-FABP level in males but did not alter L-FABP in females (Fig. 5E). While DKO increased or did not alter expression of cytotoxic cholesterol transport proteins in control-fed male (but not female) mice, the net effect in DKO cholesterol-fed female mice was to decrease the expression of proteins involved in cytotoxic transport of cholesterol for oxidation and biliary elimination, thereby overall favoring retention of cholesterol in liver.

Hepatic proteins involved in secretion of cholesterol and other lipids into serum. Assembly of neutral lipid-loaded nascent VLDL for secretion into serum requires the concerted action of L-FABP (and/or SCP-2) (12, 34, 48, 49, 66, 71, 95), MTP (82), and ApoB (82). Although hepatic levels of L-FABP and MTP were upregulated, ApoB was unaltered, in male control-fed DKO mice, while neither L-FABP, MTP, nor ApoB was increased in females (Fig. 5, E, H, and I). High-cholesterol diet alone induced expression of L-FABP (males only) but had no effect on ApoB and MTP (Fig. 5, E, H, and I). In contrast, DKO decreased L-FABP (males only), ApoB, and MTP in high-cholesterol-fed mice (Fig. 5, E, H, and I). Thus, while hepatic lipid accumulation in control-fed DKO mice was not due to loss of key proteins in VLDL assembly...
Fig. 4. Analysis of key enzymes and receptors involved in the uptake and conversion of cholesterol by quantitative real-time PCR (QrtPCR) and Western blotting. Western blots of liver homogenates isolated from WT and DKO mice were analyzed as described in EXPERIMENTAL MATERIALS to determine relative protein levels of acyl-CoA cholesterol acyltransferase-2 (ACAT2; D). Cytochrome c oxidase subunit 4 (COX4) was used as a loading control to normalize protein expression. D, inset: representative Western blot for males and females were spliced each from 1 blot, respectively and shows relative protein expression in each mouse group. QrtPCR was used to determine relative mRNA abundance of Scarb1 (A), Ldlr (B), and Ceh/Hsl (C). 18S rRNA was used to normalize mRNA expression levels. mRNA and protein expression levels were quantified as described in EXPERIMENTAL MATERIALS. Solid bars: WT mice; open bars: DKO mice. Values are means ± SE (n = 4–7). *P < 0.05 for DKO vs. WT. **P < 0.01 CH vs. CO.

Fig. 5. Analysis of key intracellular and membrane cholesterol transporters and examination of proteins involved in lipoprotein packaging by QrtPCR and Western blotting. QrtPCR was determined as in EXPERIMENTAL MATERIALS to determine relative protein levels of SCP-2 (C) and SCP-X (D). Liver fatty acid binding protein (L-FABP; E), ATP-binding cassette transporter A1 (ABCA1; F), ApoAI (G), ApoAI (H), and microsomal triglyceride transfer protein (MTP; I). COX4 was used as a loading control to normalize protein expression. E–H, inset: representative Western blots were spliced from multiple blots showing relative protein expression in each mouse group, with the same matching representative COX4 captures from the corresponding blot shown in multiple figures. Solid bars: WT mice; open bars: DKO mice. Values are means ± SE (n = 4–7). *P < 0.05 for DKO vs. WT. **P < 0.01 CH vs. CO.
tion of SREBP2 target genes in de novo cholesterol synthesis, i.e., Hmgcs1 (Fig. 7D) and Hmgcr (Fig. 7E), but not other SREBP2 target genes, i.e., Scarb1 (Fig. 4A) and Ldlr (Fig. 4B), which were unaltered. DKO did not further increase high-cholesterol diet-induced levels of SREBP2 proteins or mRNAs (Fig. 7, A–C). Likewise, DKO did not further alter transcription of other SREBP2 target genes (Figs. 4, A and B, and 7, D and E).

In summary, only in the DKO mice was the hepatic accumulation of cholesterol attributable in part to increased transcription of genes involved in de novo cholesterol synthesis. High-cholesterol diet alone or in combination with DKO did not induce de novo cholesterol synthesis genes. The potential mechanistic basis for the increased SREBP1 and SREBP2 protein is further detailed in the Discussion.

Canalicular membrane proteins involved in biliary excretion of cholesterol. L-FABP, more than SCP-2, is involved in hepatic uptake and cytosolic transfer of HDL-derived cholesterol to bile canaliculus for biliary secretion (56). Biliary hepatic uptake and cytosolic transfer of HDL-derived cholesterol to bile canaliculus for biliary secretion (56). Biliary hepatic uptake and cytosolic transfer of HDL-derived cholesterol to bile canaliculus for biliary secretion (56). Biliary hepatic uptake and cytosolic transfer of HDL-derived cholesterol to bile canaliculus for biliary secretion (56). Biliary hepatic uptake and cytosolic transfer of HDL-derived cholesterol to bile canaliculus for biliary secretion (56).

In summary, the impact of the DKO on biliary cholesterol levels was associated only in part with altered expression of canalicular cholesterol transporters, since both members of the ABCG5/ABCG8 heterodimer pairs were not concomitantly upregulated.

Hepatic, serum, and biliary bile acid levels. High-cholesterol diet alone increased hepatic, but not serum or biliary bile acid, levels in male but not female WT mice (Fig. 8, A–C). The DKO alone increased hepatic bile acid retention while not significantly altering that in serum or bile in male mice, but females were unaltered (Fig. 8A). High-cholesterol-fed DKO mice exhibited accumulation of bile acids in serum (males and females), liver (females), and bile (males) (Fig. 8, A–C). Since ~90% of total bile acid is within the bile, the total bile acid pool (liver + serum + bile) was increased only in high-cholesterol-fed male DKO mice (3,763 ± 152 nmol) compared with cholesterol-fed male WT mice (3,088 ± 269 nmol) or control-fed male DKO mice (2,948 ± 221 nmol).

Expression of proteins involved in hepatic bile acid transport. Hepatic expression of basolateral membrane (Oatp1, Oatp2, and Ntcp) bile acid transport for reuptake of bile acid...
from serum and canalicular membrane (Bsep) bile acid transporter for biliary excretion of bile acid were examined. The high-cholesterol diet alone and DKO alone in general had little overall effect on basolateral bile acid transport proteins (Fig. 8, E, F, and G) but did upregulate the canalicular Bsep, especially in high-cholesterol-fed DKO mice (Fig. 8H). In summary, the DKO-induced hepatic accumulation of bile acids was not due to concomitant massive upregulation of multiple basolateral bile acid transporters or downregulation of biliary bile acid transporter.

In contrast, because the DKO results in complete absence of both SCP-2 and SCP-x (Fig. 5, C and D), key proteins in cytosolic bile acid transport as well as bile acid synthesis, respectively, were affected. In control-fed DKO mice, this loss was compensated in part by concomitant upregulation of the other major bile acid binding/transport protein L-FABP, whose expression was increased significantly in males and tended to increase slightly in females (Fig. 5E). The cholesterol diet alone increased expression of L-FABP and SCP-x in WT males and increased that of SCP-2 and SCP-x (but not L-FABP) in WT females (Fig. 5, C–E). The DKO prevented the cholesterol diet-induced increase in hepatic L-FABP expression in males but did not alter L-FABP level in females (Fig. 5E).

Interestingly, the DKO significantly decreased hepatic expression of PCTP in DKO males and tended to decrease that in DKO females fed either diet (Fig. 8F). PCTP is the major cytosolic transport protein of phosphatidylcholine associated with phosphatidylcholine targeting to storage and/or VLDL secretion. While this change did not lead to decreased bile acid levels, it may have contributed in part to hepatic retention of phospholipids (Fig. 1C).

Expression of enzymes involved in hepatic bile acid and cholesterol synthesis. Mice and humans exhibit marked sex-dependent differential expression of the rate-limiting enzymes in the primary and secondary pathways for bile acid synthesis. Expression of CYP7A1 (rate-limiting enzyme in major/primary bile acid synthesis pathway) and CYP27A1 (rate-limiting enzyme in the alternate pathway of bile acid synthesis) was severalfold higher in female mice than male mice under all conditions examined (Fig. 9, A and B). Neither DKO alone, high-cholesterol diet alone, nor both together impacted expression of CYP7A1 in either sex (Fig. 9A). In contrast, DKO alone, but not cholesterol diet alone or both together, elicited concomitant upregulation of the alternate pathway enzyme CYP27A1 in males, while decreasing that in females (Fig. 9B). Thus the higher total bile acid level (liver + serum + bile) of cholesterol-fed, male DKO mice was not associated with concomitant upregulation of bile acid synthetic enzymes or increased levels of nuclear receptors such as PPARα, LXR, and FXR (Fig. 9, C, E, and F) that induce CYP7A1 or CYP27A1 transcription (Fig. 9, A and B).

DISCUSSION

Since mammals have a limited ability to eliminate excess cholesterol, primarily via bile, cholesterol homeostasis is closely regulated. Little is known about how the very poorly soluble cholesterol is rapidly transported and targets within the liver to regulate bile acid synthesis and secretion. Both SCP-2/SCP-x null mice and DKO mice exhibited a marked increase in hepatic cholesterol levels (Fig. 8B), although no difference in serum cholesterol levels was detected (Fig. 8A). Thus, this increased hepatic cholesterol was due to decreased hepatic secretion. The role of the primary and secondary bile acid synthetic pathways in these mice was determined, since the DKO is predicted to be deficiency in the secondary bile acid synthetic pathway.

While the DKO resulted in reduced hepatic expression of PCTP without significant alteration in serum or bile cholesterol levels, both SCP-2/SCP-x gene ablation and cholesterol-rich diet elicited concomitant increase in hepatic levels of cholesterol 7α-hydroxylase (CYP7A1; A), sterol 27-hydroxylase (CYP27A1; B), peroxisome proliferator-activated receptor-α (PPARα; C), small heterodimer partner (SHP; D), farnesoid x receptor (FXR; E), and liver x receptor (LXR; F). CYP4X was used as a loading control to normalize protein expression. Expression levels were quantified as described in EXPERIMENTAL MATERIALS. Inset: representative Western blots were spliced from multiple blots showing relative protein expression in each group, with the same matching representative COX4 captures from the corresponding blot shown in multiple figures. Solid bars: WT mice; open bars: DKO mice. Values are means ± SE (n = 5–7). *P < 0.05 for DKO vs. WT. #P < 0.05 CH vs. CO.

Fig. 9. Effect of SCP-2/SCP-x gene ablation and cholesterol-rich diet on proteins and transcription factors involved in cholesterol synthesis and oxidation to bile acids. Western blotting of homogenates isolated from the livers of DKO and WT mice were analyzed to determine relative protein levels of cholesterol 7α-hydroxylase (CYP7A1; A), sterol 27-hydroxylase (CYP27A1; B), peroxisome proliferator-activated receptor-α (PPARα; C), small heterodimer partner (SHP; D), farnesoid x receptor (FXR; E), and liver x receptor (LXR; F). COX4 was used as a loading control to normalize protein expression. Expression levels were quantified as described in EXPERIMENTAL MATERIALS. Inset: representative Western blots were spliced from multiple blots showing relative protein expression in each group, with the same matching representative COX4 captures from the corresponding blot shown in multiple figures. Solid bars: WT mice; open bars: DKO mice. Values are means ± SE (n = 5–7). *P < 0.05 for DKO vs. WT. #P < 0.05 CH vs. CO.

Fig. 8. Bile acid levels and hepatic expression of key proteins in bile acid reuptake and biliary excretion. Liver (A), serum (B), biliary (C) bile acids, and biliary cholesterol (D) levels were quantified as determined in EXPERIMENTAL MATERIALS. QRT-PCR was performed as in EXPERIMENTAL MATERIALS to determine relative mRNA abundance of Oatp1 (E), Oatp2 (F), Ntcp (G), and Bsep (H). 18S rRNA was used to normalize mRNA expression levels. Western blots of liver homogenates isolated from WT and DKO mice were obtained and analyzed as in EXPERIMENTAL MATERIALS to determine relative protein level of phosphatidylcholine transfer protein (PCTP; I), COX4 was used as a loading control to normalize protein expression. Inset: The Western blots for males and females were spliced each from 1 blot, respectively. Solid bars: WT mice; open bars: DKO mice. Values are means ± SE (n = 4–7). *P < 0.05 for DKO vs. WT. #P < 0.05 CH vs. CO.
SREBP1 and SREBP2 are released from the ER for trafficking to the ER, where they regulate the synthesis of fatty acids and cholesterol. A study involving SCP-2 and SCP-x gene ablation showed that these proteins play crucial roles in cholesterol homeostasis. In male DKO mice, cholesteryl esterification to cholesteryl ester is increased, whereas in female mice, there is no significant change. The study also demonstrates that SCP-2 facilitates cholesterol targeting to the ER, which is essential for the synthesis of mature SREBP1 and SREBP2.

First, SCP-2 facilitates cholesterol targeting to the ER, maintaining basal release of mature SREBP1 and SREBP2 in control-fed WT mice. Conversely, in the DKO mice, cholesterol accumulation is increased due to concomitant upregulation of certain enzymes involved in de novo fatty acid and cholesterol synthesis. The studies performed with whole body SCP-2/SCP-x gene ablation and high-dietary cholesterol show that hepatic lipid accumulation is regulated at least in part by SCP-2 chaperoning and high-dietary cholesterol, showing that hepatic lipid accumulation is likely due to increased de novo synthesis.

Second, SCP-2 facilitates cholesterol targeting to the ER for esterification to cholesteryl ester for metabolism in C57BL/6Ncr mice. Pathway or product decreased due to loss of SCP-2 or SCP-x (downward facing arrow), due to product increase (upward facing arrow), and loss of cholesterol transport and metabolism in male DKO mice. ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; Acc 1, acetyl-CoA carboxylase-1; Acc 2, acetyl-CoA carboxylase-2; BSEP, bile salt export pump; C, free cholesterol; CE, cholesteryl ester; CEH, cholesterol ester hydrolase; CYP27A1, cholesterol-27α-hydroxylase; ER, endoplasmic reticulum; Fas, fatty acid synthetase; Hmgcr, hydroxymethylglutaryl CoA synthase; Hmgcs, hydroxymethylglutaryl CoA reductase; INSIG, insulin-induced gene protein; LDL-R, low density lipoprotein receptor; NTCP, Na+/taurocholate cotransporting polypeptide; OATP2, organic anion transporting polypeptide 2; P, phospholipid; SCAP, SREBP cleavage activating protein; SR-B1, scavenger receptor B1; TG, triacylglycerol.

Fig. 10. Schematic diagram of proposed roles of SCP-2/SCP-x and impact of SCP-2/SCP-x gene ablation on cholesterol transport and metabolism in C57BL/6Ncr mice.
L-FABP, ApoB, and MTP) in control-fed male DKO mice. The DKO had little impact on L-FABP, ACAT2, or serum lipid levels in females.

Third, SCP-2 targets cholesterol to the ER for bile acid synthesis. SCP-2 stimulates hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme in hepatic bile acid synthesis (52, 92). Through an alternate transcription site, the SCP-2/SCP-x gene also encodes a larger protein, SCP-x (31, 90), which catalyzes the peroxisomal oxidation of cholesterol’s branched side chain to form bile acids (31, 90). Adenovirus SCP-2 overexpression increased bile acid secretion and pool size (113). However, the DKO did not decrease total bile acid (liver + serum + bile), due in part to concomitant upregulation of the other major cytosolic bile acid binding protein L-FABP, at least in male DKO mice. L-FABP not only binds bile acids (16–18, 22, 35, 50, 103), it is the most prevalent hepatic FABP family member and bile acid binding protein (22, 64).

Fourth, a high-cholesterol diet alone induced hepatic glyceride and cholesterol accumulation in the C57BL/6J WT mice, consistent with earlier high-cholesterol diet studies in both male and female mice of this strain (53, 58, 59) and with earlier studies in high-cholesterol-fed male C57BL/6J mice (21, 54). Although this high-cholesterol diet induced transcription of SREBP1 as shown herein and earlier (53), this induction was associated with a slightly decreased total mature SREBP1 in liver homogenate as shown in the male C57BL/6J strain and a decreased nuclear level of mature SREBP1 as shown earlier in the male J strain (21, 54). Likewise, although high-cholesterol diet did not alter transcription of SREBP2, the liver total mature SREBP2 was increased in the N strain mice, regardless of sex, while that appearing in the nucleus of male J strain mice was markedly decreased (21, 54). Consequently, transcription of most target genes of SREBP1 and SREBP2 was decreased more in male J strain mice (21, 54) than in the male N strain mice shown herein. Conversely, cholesterol depletion increased hepatic SREBP2 level in the hamster but surprisingly decreased SREBP1 levels of mRNA, precursor, and mature protein (93). These data indicate that cholesterol regulation of SREBP signaling is much more complex than dependence on dietary cholesterol alone, also being affected by sex, species, polyunsaturated fatty acids, oxysterols, and other factors (19, 21, 54, 93, 105, 111).

Additional contributions to the high-cholesterol diet-induced hepatic lipid accumulation include the severalfold higher cholesterol induction of hepatic L-FABP, SCP-2, and/or SCP-x (Fig. 5, E and D). Both SCP-2 (15, 44, 61, 68, 85, 87, 96, 110) and L-FABP (57) bind and preferentially enhance cholesterol transfer (SCP-2 more so than L-FABP) from plasma membranes as well as from lysosomes/lysosomal membranes to plasma membranes rather than to ER and mitochondria (3, 26, 27, 29, 30, 32, 33, 43, 65, 84, 98, 109). SCP-2 and L-FABP also increase basolateral plasma membrane cholesterol uptake/eflux (97) and target cholesterol to ER enzymes (ACAT2) for esterification to cholesterol ester (11, 13, 47, 67, 95) and/or biliary excretion (24, 70, 83, 100). SCP-x is the only known enzyme for peroxisomal oxidation of cholesterol to form bile acids (90). Taken together, the high level of L-FABP as well as SCP-2 and SCP-x in the WT C57BL/6N mice may contribute to diverting excess cholesterol to other pathways besides targeting SREBP release from the ER. However, the loss of SCP-2/SCP-x (DKO) resulted in highest hepatic SREBP1 and SREBP2 levels, especially in high-cholesterol-fed DKO mice wherein SCP-2 is ablated and in females L-FABP is down-regulated. We hypothesize that this DKO would decrease targeted delivery of cholesterol to the ER, thereby allowing increased release of mature SREBPs, as was observed (Figs. 6A and 7B). The current study examined total hepatic levels of mature SREBP1 protein and mature SREBP2 protein; however, the possibility that other factors may also contribute to nuclear distribution, retention, and efflux of these SREBPs must be considered.

Finally, it is important to note that the phenotype of control-fed DKO mice in this study differed from that reported in an earlier study with independently created male DKO mice (91). In the latter study, body weights were unaltered despite increased food intake, hepatic accumulation of cholesterol (especially cholesterol ester) as well as glycerides (phospholipid and/or triglyceride) was not observed, and hepatic levels of phospholipid were unaltered, while triglycerides decreased (91). The earlier study differed in several key aspects since their DKO mice were as follows: 1) generated using a different ablation construct strategy (91); 2) backcrossed to a different background strain (C57BL/6J rather than C57BL/6N); the J substrain differs significantly from the N in a number of genes and is much more susceptible to high-fat diet-induced obesity (reviewed in Ref. 4); and 3) the mice were fed a control diet containing high branched-chain lipid levels (i.e., 0.2 mg phytanic acid/g chow; 0.08 mg phytol/g chow) (91). In contrast, the diet in the present study was prepared phytoestrogen free and phytanic acid/phytol free, as confirmed by gas chromatography/mass spectrometric analysis, which indicated no detectable phytanic acid, and phytol was present only at 0.0028 ± 0.0004 mg/g food (data not shown). The latter difference is especially important.

Phytanic acid is a ligand for both L-FABP and PPARα (25, 41, 63), and is one of the most potent naturally occurring inducers of PPARα (1, 36, 108). L-FABP is known to transport bound fatty acids into nuclei wherein it directly interacts with (40, 42, 94, 106, 107) and facilitates ligand activation of PPARα (40, 42, 46, 74, 75, 107). Ligand induction of PPARα elicits transcription of L-FABP as well as multiple enzymes in LCFA oxidation (46, 74, 75, 89, 107). Thus the >70-fold higher dietary level of phytanic acid in the control chow of the previously generated DKO mice would be expected to significantly induce PPARα transcription of L-FABP, as confirmed by fivefold concomitant upregulation of L-FABP (28, 91), nearly three- to fivefold greater than the present study. The much higher induction of L-FABP in turn would reinforce or potentiate phytanic acid uptake and transport into the nucleus to induce PPARα transcription of LCFA oxidative enzymes and reduce hepatic levels of fatty acids acylated to phospholipid and triglyceride (91). Since phytanic acid and pristanic acid are very potent PPARα agonists that induce transcription of fatty acid oxidative enzymes and L-FABP, increased fatty acid oxidation would compensate for any increase in food consumption and thereby maintain body weight (7, 9, 20, 37). Thus the significant dietary content of branched-chain lipids (phytanic acid and phytol) in the earlier study would account for the differences in observed phenotype.

In summary, the data suggest that the SCP-2/SCP-x double gene ablation affects cholesterol transport to particular intracellular compartments with 1) less cholesterol to ER for
SREBP regulation; 2) more of what cholesterol that does arrive being used for cholesteryl ester synthesis and storage in lipid droplets; and 3) more cholesterol to peroxisomes and other organelles for bile salt synthesis and/or secretion. These data are important contributions to our understanding of regulation of sterol metabolism.

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AUTHOR CONTRIBUTIONS


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