GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Activation of Constitutive Androstane Receptor Prevents Cholesterol Gallstone Formation

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Cholesterol gallstone disease (CGD) is one of the most common gastrointestinal diseases. Lithogenic hepatic bile secretion precedes the formation of cholesterol gallstones. Constitutive androstane receptor (CAR), a member of nuclear family, plays an important role in cholesterol and bile acid metabolism. To examine whether activation of CAR can prevent cholesterol gallstone formation, we treated C57BL/6/J mice maintained on a lithogenic diet with CAR agonist 1,4-bis-[2-(3, 5-dichloropyridyloxy)] benzene and performed bile duct cannulation to study the dynamics of biliary lipids. We report that activation of CAR decreases the biliary cholesterol concentration and prevents CGD formation. The lower biliary cholesterol level was largely attributed to suppressed *Abcg5* and *Abcg8* expression in CAR-activated mice. CAR activation also promoted cholesterol conversion into bile acids by increasing the expression of *Cyp7a1*, a rate-limiting enzyme in bile acid biosynthesis. Activation of CAR enhanced bile acid re-absorption via increasing the expression of bile acid transporters *Asbt* and *Ostβ* in the ileum. The hepatic steatosis was also improved in the liver of CAR-activated mice. Furthermore, activation of CAR protected the mice against the liver X receptor α -sensitized CGD through suppressing the expression of *Abcg5/8*. Collectively, CAR plays an important role in maintaining the homeostasis of cholesterol, bile acids, and triglycerides levels, and it might be a promising therapeutic target for preventing or treating CGD. (*Am J Pathol* 2017, 187: 808–818; <http://dx.doi.org/10.1016/j.ajpath.2016.12.013>)

Cholesterol gallstone disease (CGD) is one of the most common gastroenterologic disorders. The incidence rate is increasing because of improved standards of living, a chronic high-cholesterol diet, and overnutrition. The precipitation of excess cholesterol in the bile as solid crystals is a prerequisite for cholesterol gallstone formation. In the normal physiological state, cholesterol can be solubilized in mixed micelles composed of phospholipids and bile salts, all in dynamic equilibrium. However, under certain pathophysiological conditions, especially the supersaturated bile of cholesterol, the relative excess cholesterol will precipitate as solid crystals, then aggregate, fuse, and eventually form gallstones that cause disease.¹

The main components of bile are cholesterol, phospholipids, and bile acids (BAs), which are controlled by an elaborate network of transporters. Cholesterol exits the body principally by efficient secretion into bile, mediated by the

ATP-binding cassette (Abc) transporters *Abcg5* and *Abcg8*.^{2,3} The canalicular efflux of phospholipids depends on *Abcb4*, also known as multidrug resistance protein 2 (*Mdr2*).⁴ *Abca1* is a basolateral transporter that effluxes both cholesterol and phospholipids. The primary BAs are synthesized from cholesterol in the liver controlled by multiple cytochrome P450 (Cyp) enzymes via the classical pathway (mainly mediated by *Cyp7a1* and *Cyp8b1*) or alternative pathways (mainly controlled by *Cyp7b1* and

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Cyp27a1), which accounts for approximately 75% and 25% of the total primary BAs, respectively.⁵

Cyp7a1 is the rate-limited enzyme and can be suppressed by the negative feedback regulation of BA synthesis. Cyp7a1 is down-regulated by small heterodimerizing partner (Shp) in the liver and fibroblast growth factor 15 (Fgf15) in the small intestine.^{6–8} Fgf15, derived from the small intestine, circulates in the blood to the liver, binds to the Fgf receptor 4 (Fgfr4)/ β -Klotho receptor on the surface of hepatocytes to repress the expression of Cyp7a1.⁷ When emptied from the gallbladder to the intestine, >90% of BAs can be reabsorbed by the apical sodium-dependent BA transporter (Asbt) and basolateral organic solute transporter α/β (Ost α/β) in the ileum and then circulates back to the liver through the hepatic uptake transporters sodium (Na⁺)-taurocholate cotransport protein (Ntcp) and the organic anion transporting polypeptides (Oatps).⁹ Both newly synthesized and reabsorbed BAs are secreted into the bile by the BA export pump (Bsep) and multidrug-resistance protein 2 (Mrp2). Mrp3 and Mrp4 play a role in the sinusoid export of conjugated bile salts and promote their renal secretion.¹⁰

Constitutive androstane receptor (CAR), primarily considered a xenobiotic sensor, is predominantly expressed in liver and small intestine.¹¹ It mediates specific xenobiotic induction of drug metabolism and plays an important role in stimulating BAs and bilirubin detoxification and elimination.¹² CAR activation can increase the elimination of cholesterol by its conversion into BAs and subsequent fecal excretion, with positive consequences on reverse cholesterol transport and whole-body cholesterol homeostasis.¹³ Despite the role of CAR in the elimination of BAs in liver, a recent study showed that CAR activation still maintains the biliary excretion of BAs.¹⁴ In addition, CAR plays an important role in preventing atherosclerosis.^{13,15} However, the effect of CAR activation on the pathogenesis of CGD remains unclear.

In this study, we examined activation of the nuclear receptor CAR in preventing cholesterol gallstone formation in mice. We treated C57BL6/J mice with the CAR agonist 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and simultaneously fed them with a lithogenic diet (LGD). Activation of CAR decreased the biliary cholesterol concentration and prevented CGD formation. The lower biliary cholesterol level was largely attributed to suppressed *Abcg5* and *Abcg8* expression in CAR-activated mice. CAR activation also promoted cholesterol conversion to BAs by increasing Cyp7a1 expression. Modulation of CAR activity might be a therapeutic target for CGD formation.

Materials and Methods

Animals, LGD, and Drug Treatment

All animal care and experimental procedures complied with the guidelines of the Animal Care and Utilization

Committee of the institute and were approved by this committee. Eight-week-old C57BL6/J male mice were purchased from Sichuan University Laboratory Animal center (Chengdu, China), housed with a 12:12-hour light/dark cycle, and permitted *ad libitum* consumption of water and a standard mouse diet. When necessary, mice received an intraperitoneal injection of vehicle or the CAR ligand TCPOBOP (sc-203291; Santa Cruz Biotechnology, Santa Cruz, CA) 1.0 mg/kg body weight, once per week. The vehicle- or TCPOBOP-treated mice were fed a chow diet (CD) or LGD (Harlan Teklad TD.88051) containing approximately 15.8% fat, 1.25% cholesterol, 0.5% sodium cholate, and 7.5% casein for 2 weeks. The TCPOBOP was injected 1 day before the start of LGD feeding, and the drug treatment was continued until the end of the experiments. After 2 weeks, mice were sacrificed after being deprived of food overnight, and gallbladders were removed by ligation of the cystic duct. Gallbladder bile and contents were gently squeezed into tubes after cholecystectomy and immediately examined under a polarized microscope for cholesterol crystals and gallstones. The remaining bile was stored at –20°C for biochemical analysis. Livers, gallbladders, and small intestines were frozen in liquid nitrogen and stored at –80°C. To demonstrate whether CAR activation can prevent liver X receptor (LXR) α -sensitized gallstone formation, mice were treated with intraperitoneal injection of TCPOBOP (once a week) and daily gavage of 30 mg/kg GW3965, and then fed with LGD for 2 weeks.

Bile Duct Cannulation and Collection of Hepatic Bile

Bile duct cannulation was performed as described.¹⁶ Briefly, mice were deprived of food overnight and were anesthetized with isoflurane. The lower end of the common bile duct of mice was ligated, and the common bile duct was cannulated with a PE-10 polyethylene catheter below the entrance of the cystic duct. The cystic duct was then doubly ligated, and a cholecystectomy was performed. Hepatic bile was collected in 0.2-mL tubes by gravity for 1 hour.

Histologic Evaluation and Biochemical Analysis of Bile, Hepatic Bile, Serum, Urine, and Feces

Livers and gallbladders were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin. Frozen sections (6 μ m) were used for Oil Red O staining. Images were captured by a microscope (Nikon, Tokyo, Japan). Biliary, hepatic bile, and serum BA, cholesterol, and triglyceride levels were measured with assay kits from Biosino (Beijing, China). Phospholipids were measured by using a kit from Wako (296-63801; Osaka, Japan). The cholesterol saturation index (CSI) of bile and hepatic bile was calculated according to Carey's critical tables.¹⁷ To determine urinary and fecal BA excretion, mice were individually housed, and urine and feces were collected. Fecal BAs were extracted

Figure 1 Activation of constitutive androstane receptor (CAR) prevents cholesterol gallstone disease (CGD) in C57BL/6J mice. **A:** Proportion of crystals in male C57BL/6J mice treated with vehicle (VEH) and TC (1.0 mg/kg body weight, once per week). All mice were fed a LGD for 2 weeks. The number of mice is indicated. **B:** Gross appearance of representative gallbladders. **C:** Polarizing light microscopy of cholesterol crystals. **D:** Histologic examination of gallbladder by H&E staining. The **white arrow** indicates the thickness of the gallbladder wall. The **black arrow** indicates submucosal vasodilation. $n = 5$ per group. Scale bar = 50 μm (**D**). Original magnification, $\times 100$ (**C**). H&E, hematoxylin and eosin; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

as described.¹⁸ Briefly, 0.5 g of dried feces was extracted in 10 mL of 75% ethanol at about 50°C for 2 hours, followed by centrifugation at 1500 $\times g$ for 10 minutes. BAs were then measured enzymatically in the resulting supernatant fluid. The daily feces output (in g/d per 100 g body weight) and fecal BA content (in $\mu\text{mol/d}$ per 100 g body weight) were used to calculate the rate of BA excretion. The total BA pool size was determined as BA content of the small intestine, the gallbladder, the liver, and their contents.¹⁸ After the mice were weighed, anesthetized, and exsanguinated, the fresh organs were collected, minced together, and extracted with 75% ethanol at about 50°C for 2 hours. The extract was centrifuged, BAs were determined enzymatically, and the pool size was expressed as micromoles of BAs per 100 g of body weight.

Hepatic Lipid Analysis

To analyze hepatic triglycerides and cholesterol levels, 150 mg of liver tissue was lysated into 1 mL of phosphate-buffered saline by homogenization. Total lipids were

extracted by chloroform/methanol (2:1, v/v)¹⁹ and dissolved in 1% Triton-X100 in ethanol. Triglyceride and cholesterol levels were measured by commercial assay kits from Biosino.

Hepatic Total BA Analysis

Hepatic total BAs were extracted as described.²⁰ Briefly, liver tissues were homogenized in 75% ethanol and incubated at 50°C for 2 hours. The extracted supernatant fluids were assayed by using commercial assay kits from Biosino.

Western Blot Analysis

Liver and ileum were lysed in lysis buffer and subjected to Western blot analysis with the following primary antibodies: anti-Abcg5 (dilution 1:1000; sc-25796), anti-Abcg8 (dilution 1:1000; sc-30111), and anti-Fgf15 (dilution 1:1000; sc-27177) from Santa Cruz Biotechnology; anti-Cyp7a1 (dilution 1:1000; ab-65596) from Abcam (Cambridge, MA); anti- β -actin (dilution 1:4000, A-4700) from Sigma-Aldrich (St. Louis, MO). Blots were visualized by LI-COR (Lincoln,

Figure 2 Activation of CAR alters the biochemical composition of the gallbladder and hepatic bile for antilithogenesis effects. **A–D:** Biliary concentrations of cholesterol (**A**), phospholipids (**B**), bile acids (**C**), and cholesterol saturation index (CSI) (**D**) of gallbladder bile. Male C57BL/6J mice treated with VEH and TC (1.0 mg/kg body weight, once per week). All mice were fed an LGD for 2 weeks. **E–I:** Hepatic bile flow rate (**E**) and secretory rate of cholesterol (**F**), phospholipids (**G**), bile acids (**H**), and CSI (**I**). Bile was collected from the common bile duct of 10-week-old mice. **J:** Bile acid pool size. Data are expressed as means ± SEM. $n = 5$ per group (**A–D**); $n = 7$ for each group (**E–I**); $n = 5$ for each group (**J**). * $P < 0.05$, ** $P < 0.01$. BW, body weight; CAR, constitutive androstane receptor; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

NE) Odyssey System. Quantitative determination of band intensity was conducted by Image Studio analysis software version 4.0 (LI-COR).

Quantitative Real-Time RT-PCR

Total hepatic and ileal RNA was extracted with TRIzol reagent and reverse-transcribed into complementary DNA (RR037A; TaKaRa, Kyoto, Japan). Quantitative real-time PCR (no. 170-8882AP; Bio-Rad, Hercules, CA) involved the SYBR Green-based assay with the CFX96 Real-Time system.

Statistical Analysis

Data are expressed as means ± SEM. One-way analysis of variance (Tukey's test) involved the use of GraphPad Prism version 5.0 (GraphPad Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Activation of CAR Prevents CGD in C57BL/6J Mice

To determine the effect of CAR on lithogenesis, C57BL/6J mice were fed a CD or LGD for 2 weeks with or without the

CAR agonist TCPOBOP. With a CD, vehicle- or TCPOBOP-treated mice showed no cholesterol crystals (Supplemental Figure S1A). However, with the LGD, the incidence of gallstones was 94.7% in vehicle-treated mice but decreased to 33.3% in TCPOBOP-treated mice (Figure 1A). The gallbladder in TCPOBOP-treated mice showed transparent bile, almost without any impurities compared with vehicle-treated mice that were filled with gallstones (Figure 1B). Polarizing light microscopy examination of bile revealed aggregated cholesterol crystals in vehicle-treated gallbladders, whereas TCPOBOP-treated mice were generally free of cholesterol precipitates (Figure 1C). Two-week LGD treatment caused inflammation in vehicle-treated mice, including thickening of gallbladder wall and submucosal vasodilatation. TCPOBOP treatment ameliorated these signs of inflammation (Figure 1D).

Activation of CAR Alters the Biochemical Composition of the Gallbladder and Hepatic Bile for Antilithogenesis Effects

The protective effect of CAR activation on CGD prompted us to detect the biliary biochemical composition in the gallbladder. TCPOBOP-treated mice consuming a CD had

Figure 3 Activation of CAR decreases canalicular efflux of cholesterol and increases Cyp7a1 expression. **A** and **B**: Real-time PCR analysis of hepatic mRNA expression of cholesterol canalicular transporters (**A**) and bile acid synthesis enzyme genes (**B**). **C**: Ileal mRNA expression of Shp and Fgf15. **D**: Hepatic mRNA expression of Fgfr4 and β -klotho. **E**: Hepatic level of cholesterol. **F**: Expression of hepatic Abcg5/8, Cyp7a1, and ileal Fgf15 proteins were measured by Western blot analysis. **G** and **H**: Quantitative determination of band intensity in **panel F**. Data are expressed as means $\pm$ SEM. $n = 5$ to 6 for each group. $*P < 0.05$, $**P < 0.01$. CAR, constitutive androstane receptor; CD, chow diet; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

similar biliary cholesterol, BAs, and phospholipid levels as vehicle-treated mice (Supplemental Figure S1, B–D). However, with 2 weeks of LGD feeding, TCPOBOP-treated mice showed significantly decreased biliary cholesterol concentration (Figure 2A) and slightly decreased phospholipid levels (Figure 2B). The biliary BA concentration was comparable in vehicle- and TCPOBOP-treated mice (Figure 2C). Despite its protective effect against lithogenesis, TCPOBOP treatment did not decrease biliary CSI in this phenotype (Figure 2D).

To further investigate the effect of CAR activation to prevent cholesterol gallstone formation, we performed bile duct cannulation to clarify the dynamics of bile flow and fresh biliary lipid outputs after a LGD. TCPOBOP did not change the bile flow but substantially the decreased biliary cholesterol secretion rate (Figure 2, E and F). The decreased cholesterol secretion rate was accompanied by a mild decrease in phospholipid secretion (Figure 2G) and constant BA output (Figure 2H). As a result, TCPOBOP treatment decreased CSI in hepatic bile flow (Figure 2I). The decreased hepatic biliary CSI provided a

pathophysiological explanation for the protective role of CAR activation in the lithogenic phenotype. Consistent with the similar secretion rate of BAs, total pool size of BAs in the mice of the two groups were equally comparable (Figure 2J).

Activation of CAR Decreases Canalicular Efflux of Cholesterol and Increases Cyp7a1 Expression in Liver

To gain insights into the molecular mechanisms by which CAR activation prevented cholesterol gallstone formation in C57BL6/J mice, first, we evaluated the expression levels of the genes involved in cholesterol, phospholipid, and BA transport. The mRNA expression of Cyp2b10, a target gene of CAR, was highly induced with both CD and LGD feeding after TCPOBOP treatment, indicating successful CAR activation (Supplemental Figure S2). In the liver, cholesterol is secreted by the canalicular transporters Abcg5 and Abcg8 or is converted into BAs via classic and alternative pathways that generate approximately 75% and 25% of the total primary BAs, respectively.²¹ Activation of CAR

Figure 4 Pharmacologic activation of CAR alters relative mRNA levels of genes involved in bile acid transport. **A:** Real-time PCR analysis of hepatic expression of genes involved in bile acids transport. **B:** Urinary level of bile acids. **C:** Serum level of bile acids. **D:** Ileal expression of bile acid transporters. **E:** Protein expression of ileal bile acid transporters was measured by Western blot analysis. **F:** Quantitative determination of band intensity in **E**. **G:** Fecal output of bile acids. Data are expressed as means $\pm$ SEM. $n = 5$ to 6 for each group. * $P < 0.05$, ** $P < 0.01$. CAR, constitutive androstane receptor; CD, chow diet; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

significantly decreased the mRNA and protein expression of *Abcg5* and *Abcg8* induced by the LGD (Figure 3, A, F and G), which agreed with reduced cholesterol levels in the gallbladder and hepatic bile flow. When profiling cholesterol-metabolizing genes, we found the expression of cholesterol 7 α -hydroxylase (*Cyp7a1*), a key gene involved in the classic BA synthesis pathway, was higher in the liver of TCPOBOP-treated mice (Figure 3B). LGD feeding suppressed *Cyp7a1* expression. However, this suppression of *Cyp7a1* expression was markedly reversed by CAR activation. Western blot analysis also demonstrated a significant increase in *Cyp7a1* protein expression in the liver of TCPOBOP-treated mice (Figure 3, F and G). The expression of other BA synthetic genes such as *Cyp8b1*, *Cyp7b1*, and *Cyp27a1* were not changed. This finding agreed with a previous report that overexpression of *Cyp7a1* prevented CGD.²²

To understand the mechanism of reversed *Cyp7a1* expression with TCPOBOP treatment, we measured the

expression of *Shp* and ileal *Fgf15*, two farnesoid X receptor (FXR) target genes that play an important role in negative feedback regulation of *Cyp7a1* expression.^{6–8} In the liver, *Shp* expression was unchanged (Supplemental Figure S3), suggesting the increased *Cyp7a1* expression was not caused by hepatic expression of *Shp*. The hepatic BA levels were not changed either (Supplemental Figure S4A). In the ileum, the mRNA levels of *Shp* and *Fgf15* were significantly decreased after TCPOBOP treatment (Figure 3C). *Fgf15* is secreted from the intestine into the blood circulation, then binds to the hepatic *Fgfr4*, and suppresses *Cyp7a1* expression.⁷ Activation of CAR had little effect on *Fgfr4* expression with the basal and LGD conditions (Figure 3D). However, the expression of β -klotho, a coreceptor of *Fgfr4*, was significantly inhibited in TCPOBOP-treated mice with an LGD (Figure 3D). Consistent with the mRNA level, the protein expression of *Fgf15* was also decreased as illustrated by Western blot analysis (Figure 3, F and H). The decreased *Fgf15* and β -klotho expression

may coordinately relieve the suppression of this signal on Cyp7a1 transcription. Consistent with higher conversion from cholesterol into BAs, lipid analysis revealed that the hepatic cholesterol level was significantly decreased (Figure 3E).

Activation of CAR Induces the Systematic Expression of Genes Involved in BA Transport

BAs are controlled by an elaborate network of BA transporters. In the biliary secretion system, TCPOBOP treatment had no effect on the expression of Bsep, regardless of diet, but significantly induced Mrp2 expression with a CD (Figure 4A). For sinusoid export transporters, TCPOBOP induced Mrp3 and Mrp4 mRNA levels 2.4- and 23.9-fold with a CD, respectively. When challenged with an LGD, Mrp3 and Mrp4 levels were increased 1.7- and 13.4-fold, respectively, in TCPOBOP-treated mice. This finding is consistent with the notion that Mrp4 was a target gene of CAR.²³ With a CD, TCPOBOP significantly up-regulated the expression of Oatp2 and sodium (Na⁺)-taurocholate cotransport protein, two transporters for hepatic uptake of BAs. However, activation of CAR had no effect on these two genes after an LGD. TCPOBOP treatment robustly inhibited the expression of Oatp1 regardless of the diet. Consistent with the induction of Mrp3/4, the urinary BA outputs (Figure 4B) were drastically increased in LGD-fed TCPOBOP-treated mice; however, the serum BA levels were unchanged (Figure 4C).

More than 90% of BA is reabsorbed in the ileum; therefore, we also profiled the expression of intestinal genes involved in BA transport. The LGD significantly suppressed the expression of Asbt regardless of drug treatment (Figure 4D). TCPOBOP treatment did not affect Asbt mRNA expression compared with vehicle treatment. TCPOBOP increased Ost β mRNA expression with a CD but not with a LGD. The LGD markedly decreased the Mrp3 mRNA level in TCPOBOP-treated mice. The mRNA expression of Ost α was unchanged among the four groups of mice. Although no changes were observed in the mRNA expression of these ileal BA transporters, Western blot analysis revealed that TCPOBOP dramatically increased Asbt and Ost β protein expression in LGD-fed mice (Figure 4, E and F). It indicated that activation of CAR increased BA reabsorption in the ileum of the mice consuming a LGD. Consistent with these facts, we also found the average fecal BA excretion was significantly reduced in TCPOBOP-treated mice at day 9 of the LGD (Figure 4G).

Activation of CAR Decreases the Expression of Genes Involved in Phospholipid Transport

Although activation of CAR protected mice against CGD, we observed decreased phospholipid secretion into the gallbladder (Figure 2G). The level of Abcb4, a major

Figure 5 Activation of CAR decreases the expression of phospholipid transporters. Real-time PCR analysis of hepatic mRNA expression of phospholipid transporters. Data are expressed as means $\pm$ SEM. $n = 5$ to 6 for each group * $P < 0.05$, ** $P < 0.01$. CAR, constitutive androstane receptor; CD, chow diet; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

phospholipid transporter, was markedly decreased with both a CD and LGD in TCPOBOP-treated animals (Figure 5). The level of Abca1 was slightly increased with a CD but unchanged with an LGD.

Activation of CAR Decreases the Expression of Genes Involved in Lipogenesis

CAR is an antiobesity nuclear receptor.²⁴ In our study, TCPOBOP treatment significantly decreased the expression of a gene involved in lipogenesis, sterol regulatory element-binding protein (Srebp-1c), regardless of diet. The expression of stearoyl-CoA desaturase-1 (Scd-1) was unregulated with a CD but markedly suppressed with an LGD (Figure 6A). TCPOBOP did not change hepatic tissue architecture in mice maintained on a CD (Figure 6B). With an LGD, the livers of vehicle-treated mice were filled with large lipid droplets by evidence of both macrovesicular and microvesicular steatosis. However, TCPOBOP-treated mice showed less lipid accumulation, which was further illustrated by Oil Red O staining of the liver (Figure 6C). Biochemical analysis revealed that, as expected, LGD feeding increased hepatic triglyceride levels, and TCPOBOP treatment decreased hepatic triglyceride content both with a CD and LGD compared with vehicle-treated mice (Figure 6D). Similarly, serum triglycerides were decreased in TCPOBOP-treated mice, whereas the circulating cholesterol was slightly increased (Supplemental Figure S4B).

Figure 6 Pharmacologic activation of CAR decreases hepatic steatosis. **A:** Real-time PCR analysis of hepatic mRNA expression of genes involved in lipogenesis. **B:** Representative images of H&E-stained sections of livers. **Arrows** indicate macrovesicular and microvesicular steatosis. **C:** Oil Red O staining of liver sections. **D:** Analysis of hepatic triglycerides level. Data are expressed as means $\pm$ SEM. $n = 5$ to 6 for each group (**A** and **D**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 50 μ m (**B** and **C**). CAR, constitutive androstane receptor; CD, chow diet; H&E, hematoxylin and eosin; LGD, lithogenic diet; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

Activation of CAR Prevents the LXR α -Sensitized Gallstone Disease

It was reported that activation of LXR α sensitized mice to gallbladder cholesterol crystallization by increasing cholesterol transport to the gallbladder.²⁵ We were interested in whether CAR activation can attenuate gallbladder cholesterol crystallization caused by LXR α agonist *in vivo*. Activation of LXR α sensitized mice to gallstone disease, similar to a previous report.²⁵ However, TCPOBOP treatment attenuated the aggravated gallbladder cholesterol crystallization induced by GW3965 (Figure 7, A and B). The cholesterol transporters Abcg5 and Abcg8 were highly induced by LXR α (Figure 7C). However, this induction was significantly inhibited by CAR activation (Figure 7C).

Discussion

Previous studies have shown that CAR played important roles in reverse cholesterol transport, atherosclerosis, and BA elimination.^{13,15,26} However, the role of CAR in CGD is still unknown. In this study, we showed that activation of CAR prevented cholesterol gallstone formation in mice consuming an LGD. The protective role was associated with the markedly decreased secretion of cholesterol into the gallbladder. Although the CSI of bile in the gallbladder did not change, the prevalence of cholesterol gallstones was markedly decreased in TCPOBOP-treated mice. In the liver,

the relative greatly decreased biliary cholesterol level and slightly decreased phospholipid secretion, combined with unchanged BA secretion, contributed to the decreased CSI of hepatic fresh bile, thus being antilithogenic. Activation of CAR also protected mice against LXR α -sensitized gallstone formation. Although we showed that the activation of CAR by TCPOBOP had beneficial effects with the CGD, this study is limited because CAR knockout mice were not used to evaluate whether TCPOBOP effect is CAR dependent. In addition, CAR knockout mice should be used to determine whether ablation of this receptor sensitized mice to gallstone disease in the future.

Recently, the role of CAR in cholesterol transport was highlighted, especially the role in reverse cholesterol transport and atherosclerosis. Therefore, CAR may play an important role in whole-body cholesterol homeostasis. Indeed, activation of CAR can decrease high-density lipoprotein cholesterol level, the main component in the reverse transport of cholesterol to the liver.²⁷ However, CAR activation can also stimulate reverse cholesterol transport and can protect mice against atherosclerosis along with decreased biliary cholesterol level by repressing the expression of the Abcg5/8 cholesterol transporters.¹³ In agreement, we found decreased biliary secretion of cholesterol into the gallbladder of TCPOBOP-treated mice as well as the significantly suppressed mRNA and protein levels of Abcg5/8, which are required for essential secretion of cholesterol into bile. In addition, we observed a slight reduction in phospholipid secretion in bile of TCPOBOP-treated mice. This finding

could be explained by the decreased biliary export of cholesterol. Abcg5/8 required Abcb4 for efficient secretion of cholesterol from liver into bile.²⁸ We also found that altered hepatic secretion of biliary lipids is a prerequisite for cholesterol supersaturated bile in the gallbladder and

subsequent cholesterol gallstone formation.²⁹ Therefore, the decreased CSI of the hepatic bile in TCPOBOP-treated mice precedes the effect of CAR on protecting cholesterol gallstone formation, for a pathophysiological explanation of the protective role of CAR activation in the lithogenic phenotype.

We found that an LGD markedly inhibited the expression of Cyp7a1 (the rate-limiting enzyme of BA biosynthesis), which was consistent with a previous study.²⁵ This finding is due to the presence of BAs in the LGD, which may signal the negative feedback mechanism of regulating BAs.³⁰ The expression of Cyp7a1 was significantly higher in TCPOBOP-treated mice than in vehicle-treated mice regardless of diet. Miao et al³¹ reported that activation of CAR inhibited Cyp7a1 expression, which is opposite to our results. We reasoned this discrepancy may be the result of different mouse strains used. BALB/c mice were used in their study, whereas we used C57BL/6J mice. It is also possible that a different dosage of TCPOBOP might have a different effect on Cyp7a1 expression (TCPOBOP 0.3 mg/kg body weight in their study and 1.0 mg/kg body weight in ours). Cyp7a1 is negatively regulated by Shp signaling in the liver³² and Fgf15 from the intestine.⁷ We found the hepatic expression of Shp did not change, which agreed with the constant hepatic BA level (Supplemental Figure S4A). However, the ileal expression of Shp and Fgf15 was significantly suppressed by the TCPOBOP treatment. The lower level of Fgf15 could weaken negative feedback and thereby resulted in higher expression of Cyp7a1.

Previous studies have shown that CAR played an important role in xenobiotic molecular elimination and also controlled the metabolism of endogenous compounds such as BAs and triglycerides.^{26,33,34} Despite the activation of Cyp7a1, our TCPOBOP-treated mice showed a constant biliary level of BAs, which was due to the unchanged Bsep and Mrp2 expression that mediated canalicular BA efflux. With increased synthesis of BAs in the liver, CAR activation decreased hepatic BA uptake. TCPOBOP treatment robustly suppressed the expression of Oatp1, thereby indicating a decreased hepatic Na⁺-independent import of BAs. The increased BA synthesis but constant biliary BA level in TCPOBOP-treated mice might be due to the overwhelming up-regulation of Mrp4 (ABCC4), a target gene of CAR, which is responsible for the sinusoidal efflux of BAs from the hepatocytes into the space of Disse.^{23,35} When mice were maintained on a western-type diet, Sberna et al¹³ found

Figure 7 Activation of CAR prevents the LXR α -sensitized gallstone disease. **A:** Gross appearance of representative gallbladders in C57BL/6J mice treated with VEH, TC (1.0 mg/kg body weight, once per week), and/or GW (30 mg/kg body weight, once per day). All mice were fed a LGD for 2 weeks. **B:** Polarizing light microscopy of cholesterol crystals. **C:** Real-time PCR analysis of hepatic mRNA expression of cholesterol transporters Abcg5 and Abcg8. Data are expressed as means $\pm$ SEM. $n = 5$ for each group. * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 100$ (B). CAR, constitutive androstane receptor; CD, chow diet; GW, GW3965; LGD, lithogenic diet; LXR α , liver X receptor α ; TC, TCPOBOP or 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; VEH, vehicle.

that activation of CAR by TCPOBOP stimulated fecal BA excretion. In our results, TCPOBOP treatment suppressed the fecal excretion of BAs. In their study, CAR activation increased the sinusoidal efflux and enhanced the hepatic BA uptake. In addition, the canalicular membrane secretion of BAs was significantly increased, which is essential to the stimulated fecal excretion of BAs. In our study, we found that the protein levels of ileal BA transporters Asbt and Ost β were significantly higher in TCPOBOP-treated mice (Figure 4, E and F). The higher expression of Asbt and Ost β will increase the reabsorption of BA and subsequently decrease fecal BA excretion. We reasoned this discrepancy might be due to the presence of cholate in the diet. The cholate will signal a negative feedback on Asbt, Ost α , and Ost β expression. However, this negative feedback might be relieved when CAR is activated.

Several nuclear receptors affect lithogenesis and include FXR, LXR, pregnane X receptor (PXR), and estrogen receptors.^{10,25,36–38} We found that CAR played an anti-lithogenic role in lithogenesis similar to FXR and PXR. However, the mechanisms were quite different. CAR activation played a central role in canalicular membrane transport of cholesterol, whereas FXR activation had profound effects on canalicular BA and phospholipid secretion.³⁶ In addition, TCPOBOP induced the conversion from cholesterol into BAs, whereas FXR suppressed the expression of Cyp7a1.

Here, we demonstrated that activation of CAR by TCPOBOP prevents cholesterol gallstone formation via inhibiting cholesterol transport from the liver to the gallbladder. PXR, a sister xenobiotic receptor of CAR, also prevents CGD.¹⁰ Like in the case of drug metabolism, the function of these two xenobiotic receptors in CGD is also conserved. CAR and PXR share many of their target genes and indirectly regulated genes. Activation of PXR prevented CGD by decreasing biliary concentrations of cholesterol and increasing the level of bile salts. In contrast to PXR, activation of CAR prevented CGD by decreasing biliary concentrations of cholesterol without changing the level of bile salts. Mechanistically, both receptors decreased cholesterol secretion through inhibiting the expression of Abcg5/8. In contrast to the unchanged biliary BA level in CAR-activated mice, PXR activation increased biliary BAs via induction of Mrp2 without Bsep. In the intestine, both PXR and CAR increased BAs reabsorption by increasing Asbt expression. Thus, both receptors provide a rationale for pharmacologic interference with CGD via these nuclear receptors. The present and previous studies establish a crucial and collective role for PXR and CAR in preventing CGD *in vivo*.¹⁰ We conclude that CAR and PXR mediate overlapping yet distinct defense to prevent CGD. Combined loss of PXR and CAR heightens the sensitivity to toxic BAs in mice.³⁹ It is also tempting to speculate that a combined defect in PXR and CAR expression and/or activation may increase and/or decrease susceptibility to CGD and may present a novel research topic.

LXR α has a lithogenic effect opposite to that of CAR. The lithogenic effect of LXR α was due to the combined effect of increased biliary cholesterol and phospholipids and decreased biliary BAs.²⁵ In our study, we found markedly decreased hepatic secretion of cholesterol, slightly decreased phospholipid secretion, and unchanged BA secretion. Activation of LXR α suppresses CAR-mediated xenobiotic response, and, conversely, activation of CAR inhibits the LXR α -mediated lipogenesis.⁴⁰ Indeed, we observed that the hepatic triglyceride level was markedly decreased after a 2-week TCPOBOP treatment, probably because of the suppressed expression of Srebp-1c and Scd-1 in the liver. Activation of CAR also protected the mice from LXR α -sensitized gallstone disease by suppressing the expression of Abcg5 and Abcg8 induced by LXR α . Our findings expand the cognitive territory of the checks and balances behind LXR α and CAR.

Conclusions

We provide evidence that CAR may be a new factor in preventing cholesterol gallstone formation in a mouse model under lithogenic conditions. Activation of CAR may represent a new approach for treating CGD. Certainly, whether CAR could be a novel target in preventing or treating CGD in humans remains to be further determined.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.12.013>.

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