Disruption of the Murine Protein Kinase Cβ Gene Promotes Gallstone Formation and Alters Biliary Lipid and Hepatic Cholesterol Metabolism*

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The protein kinase C (PKC) family of Ca2+ and/or lipid-activated serine-threonine protein kinases is implicated in the pathogenesis of obesity and insulin resistance. We recently reported that protein kinase Cβ (PKCβ), a calcium-, diacylglycerol-, and phospholipid-dependent kinase, is critical for maintaining whole body triglyceride homeostasis. We now report that PKCβ deficiency has profound effects on murine hepatic cholesterol metabolism, including hypersensitivity to diet-induced gallstone formation. The incidence of gallstones increased from 9% in control mice to 95% in PKCβ−/− mice. Gallstone formation in the mutant mice was accompanied by hyposecretion of bile acids with no alteration in fecal bile acid excretion, increased biliary cholesterol saturation and hydrophobicity indices, as well as hepatic p42/44MAPK activation, all of which enhance susceptibility to gallstone formation. Lithogenic diet-fed PKCβ−/− mice also displayed decreased expression of hepatic cholesterol-7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8b1). Finally, feeding a modified lithogenic diet supplemented with milk fat, instead of cocoa butter, both increased the severity of and shortened the interval for gallstone formation in PKCβ−/− mice and was associated with dramatic increases in cholesterol saturation and hydrophobicity indices. Taken together, the findings reveal a hitherto unrecognized role of PKCβ in fine tuning diet-induced cholesterol and bile acid homeostasis, thus identifying PKCβ as a major physiological regulator of both triglyceride and cholesterol homeostasis.

Gallstone disease is a major public health problem in all developed countries. Approximately 12% of individuals in the United States are affected by gallstones with more than 750,000 cholecystectomies being performed each year (1,2). The pathogenesis of gallstone disease is multifactorial, involving interactions between genetic and environmental factors that result in bile supersaturation, gallbladder hypomotility, and precipitation/nucleation of cholesterol microcrystals (3,4). Obesity, aging, estrogen treatment, and diabetes are consistently associated with a higher risk of gallstones (5,6). Interestingly, individuals undergoing weight loss also have a high risk of developing gallstones (7–9). The signaling mechanisms that link changes in the triglyceride (TG)3 content of the body and impaired glucose metabolism to cholesterol gallstone formation are unknown, but our knowledge is rapidly expanding. Systemic genomewide scans in mice from experimental crosses of inbred mouse strains have mapped the “Lith” loci to increased gallstone susceptibility (3,4). A growing number of genetic variants in some of these genes have been linked to human gallstone disease (10).

Protein kinase C (PKC) family members are lipid-dependent serine/threonine protein kinases that play key roles in a wide spectrum of signal transduction pathways and are of major interest as therapeutic targets for human diseases (11,12). There are 12 family members that can be classified on the basis of their structures into three groups: classic (α, βI, βII, and γ), novel (δ, ε, η, and τ), and atypical (λ and θ). The exact function of the different PKC isoforms is not yet established. However, there is evidence that individual PKC isoforms show different substrate specificities, subcellular localization, cofactor requirements, and sensitivity to agonist-induced down-regulation (13). Previous studies have firmly established that diabetic conditions activate the diacylglycerol-PKC signaling pathway (14–16). The induction appears to be restricted to a few “diabetes-related” isoforms in a tissue-specific manner. PKCβ is one of these isoforms and has been most directly linked to important aspects of hyperglycemia in vivo and in vitro (15). Interestingly, PKCβ was one of the earliest isoforms recognized in insulin signaling and appears to play dual roles in insulin signaling (17). PKCβ is involved in the transduction of specific insulin signals but also contributes to the generation of insulin resistance. For instance, PKCβ is activated by insulin and acts upstream of PI 3-kinase and mitogen-activated protein kinase (18). In contrast, activation of PKCβ results in increased serine/ threonine kinase activity, which enhances the expression of PPARα, a peroxisome proliferator-activated receptor α.

3 The abbreviations used are: TG, triglyceride(s); CSI, cholesterol saturation index; CYP7A1, cholesterol 7α-hydroxylase; CYP8b1, sterol 12α-hydroxylase; MMLD, modified milk-fat-containing lithogenic diet; PLD, purified lithogenic diet; WAT, white adipose tissue; LXRx, liver X receptor; FXR, farnesoid X receptor; SHP, small heterodimer protein; PPARα, peroxisome proliferator-activated receptor α.
threonine phosphorylation of insulin receptor substrate-1 (19), and its excessive phosphorylation has been proposed to be one of the mechanisms whereby dietary lipids and tumor necrosis factor induce insulin resistance (20, 21).

We recently reported that PKCβ-deficient (PKCβ−/−) mice are lean and exhibit resistance to high fat diet-induced obesity, hepatic steatosis, and insulin resistance (22, 23). Interestingly, these mice show hypocholesterolemia in response to a high fat diet, suggesting an influence of PKCβ in the regulation of cholesterol homeostasis. In addition, several previous studies have also indicated a role for PKC isoforms in bile acid homeostasis. For example, cholesterol sulfate has been shown to activate specific PKC isoforms, including PKCβ (24, 25). In addition, tauoursodeoxycholic acid, which is used in the treatment of cholestatic liver disease, is known to stimulate biliary secretion of bile acids through Ca2+-dependent PKC activation (26). Furthermore, PKCβ (chromosome 7, 117.5 Mb, 61.0 centimorgans) is a positional candidate for the H9252 gene that was detected on chromosome 7 with peak linkage at 60 centimorgans (27, 28). The potential effects of PKCβ on insulin signaling, obesity, cholesterol, and TG homeostasis led us to hypothesize that PKCβ may modify the pathogenesis of diet-induced gallstone formation.

In the present study, we explored the in vivo role of PKCβ in diet-induced gallstone formation using PKCβ−/− mice. Our data show that PKCβ-deficient mice have greatly enhanced sensitivity to diet-induced gallstones despite their apparent protection from high fat diet-induced obesity and insulin resistance. We propose that PKCβ plays a pivotal role in the metabolic adaptation to lithogenic diet to prevent gallstone formation.

EXPERIMENTAL PROCEDURES

Animals and Lithogenic Diet—PKCβ-deficient C57BL/6J mice have been described earlier (22, 23). Eight-week-old male wild-type (WT) and PKCβ−/− mice were fed ad libitum for 7–8 weeks either a purified lithogenic diet (PLD) (TD-04428, Harlan Teklad, Madison, WI) containing 19% fat (cocoa butter is the primary fat source), 1% cholesterol, 0.5% cholic acid, and essential minerals and vitamins or a standard chow diet containing 8% fat (7912 rodent chow; Harlan Teklad). To study effects related to the substitution of cocoa butter with fat of animal origin, lithogenic diet composition was the same except for the substitution of cocoa butter for canola oil. Animals were housed under controlled temperature (23 °C) and lighting (12-h light/dark cycle) conditions with free access to water. C57BL/6J WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Unless otherwise indicated, all experiments were performed on male animals that were fasted for ~16 h. At the indicated period, animals were killed, cholecystectomy was performed, and gallbladders were examined for cholesterol gallstones. The presence of gallstones was determined by gross examination of the gallbladder with the naked eye. All experiments were approved by the Institutional Animal Care and Research Advisory Committee of the Ohio State University College of Medicine.

Lipid and Lipoprotein Analyses—Plasma TG, tissue TG, and cholesterol concentrations were measured by colorimetric assays (Roche Diagnostics). Serum leptin and adiponectin levels were measured by enzyme-linked immunosorbent assay kits (Linco Inc.) according to the manufacturer’s protocol. Biliary (10 μl) and hepatic (0.2 g of liver) lipids were extracted according to the Folch method (29). For comparative analysis of tissue lipids, lipids were extracted from liver, separated with ALISIL silica gel TLC plates (Whatman) using hexane/ethyl ether/acetone (83:16:1), and stained with iodine vapor (30). Phospholipids were measured as inorganic phosphorus, and bile salts were enzymatically quantified using the 3α-hydroxysteroid dehydrogenase method (Diazyme Laboratories, Poway, CA) (31). Analysis of lipids in lipoprotein fractions was performed after separating pooled plasma samples by fast-performance liquid chromatography (FPLC) with a Superose 6 10/300 GL high performance column (GE Healthcare LifeScience) by personnel in the Mouse Metabolic Phenotyping Center facility at the University of Cincinnati College of Medicine. Fractions were assayed for total cholesterol and triglycerides. Plasma alanine aminotransferase and aspartate aminotransferase activities were measured with the International Federation of Clinical Chemistry reference method without pyridoxal phosphate activation by personnel in the chemistry laboratories at the Veterinary Hospital of the Ohio State University.

Histological Analysis of Tissues—Liver, white adipose tissue (WAT), and gallbladder were isolated from WT and PKCβ−/− mice. Tissues were fixed with 4% formaldehyde in phosphate-buffered saline, processed, and stained with hematoxylin-eosin.

Bile Flow, Biliary Lipid Output, and Bile Acid Species Determination—Mice were anesthetized after a 4-h fast. An external bile fistula was established surgically via the gallbladder fundus. Hepatic bile was collected for up to 60 min while maintaining body temperature at 37 °C. Hepatic bile volume was determined gravimetrically, assuming a density of 1 g/ml. Bile samples were stored at −80 °C until analyzed. Biliary phospholipids and cholesterol were determined enzymatically using a phospholipids B kit and a cholesterol E kit from Wako Chemicals (32, 33). Cholesterol saturation indices in hepatic bile were calculated using published parameters (34). Total bile acid content and individual bile acid concentrations were measured via high performance liquid chromatography (32, 33). In brief, 100 μl of diluted hepatic bile (1:20 dilution prepared in methanol) was injected onto a Pursuit C18 reverse phase column (Varian, Lake Forest, CA) using a Rainin HPXL HPLC system (Varian). Bile acid standards were purchased from Steraloids Inc. (Newport, RI) or from Calbiochem. The hydrophobic index of hepatic bile samples was calculated as described earlier (32, 33).

Bile Acid Pool Size and Fecal Bile Acid Output Determination—Bile acid pool size was determined as the sum total bile acid content of the entire small intestine, gallbladder, and liver, which were homogenized and extracted together in ethanol (32). Total bile acid mass was determined enzymatically. In addition, fecal bile acid output was determined from stool quantitatively collected from individually housed mice for periods up to 72 h. For each determination, 100-mg duplicate ali-
quot of dried feces were extracted into ethanol as described (32), and total bile acid mass was again measured enzymatically.

RNA Extraction and Gene Expression Analyses—We obtained liver samples from lithogenic diet-fed mice (16 h after starvation). Total RNA was extracted with TRIzol (Invitrogen), and samples were pooled (n = 5) for analysis as described earlier (22, 23).

Microsome Preparation—Livers from mice of the indicated genotype were quickly removed and homogenized at 4 °C in buffer I (40 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, 50 mM KCl, 50 mM KF, 300 mM sucrose, and 1 × protease inhibitor mixture. The homogenate was centrifuged at 20,000 × g for 20 min, and the supernatant was further centrifuged at 108,000 × g for 60 min. The pellet was washed and resuspended in buffer II (buffer I without sucrose), and aliquots representing 80 μg of microsomal protein were separated by 10% SDS-PAGE. Western blotting was performed with the anti-mouse CYP7A1 antibody (Cosmo Bio USA, Inc., Carlsbad, CA).

Western Blotting—Tissue samples were snap-frozen, pulverized, and dispersed in lysis buffer. Samples were loaded onto 12% acrylamide gels and blotted. Blots were probed using antiphospho-p42/44 MAPK or phosphorylation-independent antibodies. Immunoreactive proteins were visualized via enhanced chemiluminescence (ECL Plus; Amersham Biosciences) and quantified via densitometry using the Molecular Analyst software (Bio-Rad).

TABLE 1
Comparison of gallstone formation between genotypes

| Genotype | Diet | Gallstones |
|----------|------|------------|
| WT       | Chow | 0% (0/22)  |
| PKCβ⁻/⁻  | Chow | 0% (0/22)  |
| WT       | PLD  | 9% (2/22)  |
| PKCβ⁻/⁻  | PLD  | 95% (21/22)|

Statistical Analysis—Statistical comparisons were performed using unpaired Student’s t tests with p < 0.05 being considered statistically significant.

RESULTS

PKCβ Deficiency Sensitizes Mice to Lithogenic Diet-induced Gallstone Formation—To study the susceptibility of PKCβ⁻/⁻ mice to gallstone formation, we fed a PLD for 8 weeks and measured body weight, food intake, and gallstone formation. As expected from our previous work, WT mice were on average 2.5 g heavier than PKCβ⁻/⁻ mice, although the food intake of PKCβ⁻/⁻ mice was 20% higher than that of WT mice during the feeding study (Fig. 1, A and B). There were no discernable gallstones in the gallbladders from either genotype when fed a chow diet for 8 weeks (Table 1). However, in WT mice fed a lithogenic diet, 9% of gallbladders exhibited grossly visible gallstones. In marked contrast, almost all gallbladders (95%) from PKCβ⁻/⁻ mice fed this diet were engorged with gallstones to varying degrees (Fig. 1C). Table 1 shows semiquantitative analysis of cholesterol gallstone formation in WT and PKCβ⁻/⁻
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![Comparison of metabolic profiles of WT and PKCβ−/− mice fed PLD.](image)

FIGURE 2. Comparison of metabolic profiles of WT and PKCβ−/− mice fed PLD. A, plasma cholesterol and TG levels. B, cholesterol- and TG-lipoprotein distributions are altered by PKCβ deficiency in C57BL/6J mice on the PLD. Lipoprotein cholesterol and TG distributions were determined in pooled serum from five individual mice/group. Open shapes, WT mice; closed shapes, PKCβ−/− mice. IDL, intermediate density lipoprotein. C, relative amounts of liver TG and cholesterol (CE) contents following PLD feeding for 8 weeks. Each value represents the mean ± S.D. (n = 5–10 mice/group). Thin layer chromatography of total lipid extract from the livers of WT and PKCβ−/− mice is also shown. Each lane represents lipids from the livers of individual mice. *, p < 0.05.

mice fed PLD. The low incidence of gallstone formation in WT mice observed in our study is mainly attributed to the feeding of a semisynthetic and refined PLD as compared with lithogenic diets used in other studies (35, 36). Because the fat content is derived primarily from cocoa butter, this purified diet has been shown to consistently reduce liver damage and gallstone formation in this strain (37, 38).

There was a 28% increase in plasma cholesterol concentrations and a 39% decrease in plasma TG concentrations in PKCβ−/− mice as compared with WT mice fed PLD (Fig. 2A). Fig. 2B shows the lipoprotein profile of the fresh plasma samples obtained from each experimental group of mice. VLDL, intermediate density lipoprotein, and HDL TG were significantly low in PKCβ−/− mice; however, these mice exhibited a lipoprotein cholesterol distribution that had a slight increase in intermediate density lipoprotein/LDL fractions, possibly reflecting the replacement of TG in the core of apoB-containing lipoproteins with cholesterol esters. Marked differences in hepatic TG contents were also observed between the WT and PKCβ−/− mice (Fig. 2C). PKCβ−/− mice exhibited significantly lower hepatic TG content, whereas hepatic total cholesterol content was 29% higher (Fig. 2C).

We also examined liver and fat histology and measured adipokine levels. The PLD did not cause any significant differences in liver weights between WT and PKCβ−/− mice (Fig. 3A), and no obvious histological liver damage was observed in PKCβ−/− mice (Fig. 3B). Neither alanine aminotransferase nor aspartate aminotransferase activities were significantly different between WT and PKCβ−/− mice (data not shown). In contrast, epididymal WAT weights of PKCβ−/− mice were significantly lower (~2.5-fold) than those in control mice (Fig. 3A), but the weights of other organs, such as the kidney and muscles, did not differ (results not shown). Histological analysis of WAT showed that the size of lipid droplets in adipocytes was generally smaller in PKCβ−/− mice than in controls (Fig. 3B), suggesting that the decreased cell size partly contributed to the fat mass decrease. Consistent with adipose tissue mass, PKCβ−/− mice fed a lithogenic diet exhibited significantly lower serum leptin levels, whereas serum adiponectin levels were unchanged (Fig. 3C). In addition, gallbladder volume and turbidity were increased in PKCβ−/− mice versus WT mice (Fig. 3D). The gallbladders from PKCβ−/− mice showed wall thickening and submucosal vasodilation (Fig. 3D).

Alterations in Biliary Lipid Profiles in PKCβ−/− Mice on Chow or Lithogenic Diet—The lithogenic phenotype prompted us to analyze the biliary lipid rates and lipid composition. Comparable bile flow rates were observed in chow-fed mice of both genotypes with comparable secretion rates of cholesterol (Fig. 4, A and D). By contrast, there was a trend to reduced phospholipid secretion but a significant decrease in bile acid secretion rates in PKCβ−/− mice (Fig. 4B), which was accompanied by a significant increase in cholesterol saturation index (CSI) (Fig. 4C). On the other hand, biliary concentrations of cholesterol, phospholipids, and bile salt were significantly higher in PLD-fed PKCβ−/− mice (Fig. 4E), but no significant difference was observed in biliary CSI (Fig. 4F). Individual bile acid species determination by HPLC revealed that the primary bile acids β-muricholate and taurocholate together represent the domi-
nant species in chow-fed animals of both genotypes (Fig. 5A), without alterations to the hydrophobicity index of bile (Fig. 5C). In response to lithogenic feeding, there was a shift in the bile acid species, with taurocholate representing the major species with a corresponding decrease in β-muricholate for both genotypes (Fig. 5A). In addition, there was a further increase in the relative abundance of taurodeoxycholate in PKCβ−/− mice (Fig. 5B). There was a significant increase in hydrophobicity in PKCβ−/− bile upon lithogenic feeding (Fig. 5D). Increases in either CSI or hydrophobicity are known to facilitate cholesterol precipitation (39), providing a plausible biochemical explanation for the lithogenic phenotype of PKCβ−/− mice.

Bile Acid Pool Size and Synthetic Rates in PKCβ−/− Mice—To better understand the general bile acid homeostasis, we also eval-

FIGURE 3. Feeding PLD did not cause any major hepatotoxicity. A, weights of liver and WAT for WT and PKCβ−/− mice fed PLD diet for 8 weeks (n = 5 for each genotype). BW, body weight. B, liver and WAT histology (hematoxylin-eosin staining) of the same group of mice. Pictures are representative of 8–10 images obtained per animal in groups of 3–4 animals. This experiment was performed 2–3 times. Magnification: ×20. C, serum leptin and adiponectin levels in these mice. Each value represents the mean ± S.D. (n = 5–10 mice/group), *, p < 0.05; **, p < 0.01. D, gallbladder volumes and histology from each genotype.

FIGURE 4. Biliary flow, lipid secretion rates, and hepatic bile cholesterol saturation indices on chow or PLD. Hepatic bile was collected as described for 60 min. A and D, biliary flow rates (μl/min/kg of body weight). B and E, biliary bile salt secretion (μmol/min/kg of body weight). C and F, hepatic bile CSIs were calculated from the parameters in panels B and E using Carey’s critical tables. Bar graphs show the mean ± S.E. (n = 4–8 for each genotype), *, p < 0.05.
uated the total bile acid pool size and fecal excretion. We observed comparable total bile acid pool size in chow-fed WT and PKCβ−/− mice (Fig. 6A), and fecal bile acid excretion rates (at steady state, a surrogate for bile acid synthesis rates) revealed no statistically significant difference between the genotypes. By contrast, upon feeding the PLD, we observed a 1.4-fold increase in the bile acid pool size in PKCβ−/− mice (Fig. 6A), which was not accompanied by any alteration in fecal bile acid output (Fig. 6B). The expanded bile acid pool (largely the result of dietary cholate supplementation) in the setting of any alteration in fecal losses suggests the possibility that enterohepatic bile acid recycling is increased in PKCβ−/− mice. This possibility is also supported by the aforementioned changes in chow-fed PKCβ−/− mice (Fig. 4B), where decreased bile acid secretion rates in the setting of unchanged bile acid pool size or fecal bile acid excretion (Fig. 6, A and B) again imply that alterations in enterohepatic cycling may contribute to this phenotype.

**Substitution of Cocoa Butter to Milk Fat Shortened Time Required for Gallstone Formation in PKCβ−/− Mice**—To begin to understand the influence of individual dietary components on increased lithogenicity of PKCβ−/− mice, we turned to an MMLD in which cocoa butter was replaced with a fat of animal origin (anhydrous milk fat). Cocoa butter (57.8% saturated, 34.6% monounsaturated, and 7.6% polyunsaturated fatty acids)
is comparable in fatty acid composition with milk fat (55.2% saturated, 29.8% monounsaturated, and 15.1% polyunsaturated fatty acids). However, the actual percentage of specific types of fatty acids differed between these diets. For example, 31% of saturated fatty acids in cocoa butter came from stearic acid, whereas it accounted for only 10% of saturated fatty acids in milk fat.

WT and PKCβ−/− mice were fed MMLD that contained 1% cholesterol, 0.5% cholate, and 15% fat (anhydrous milk fat) for 4 weeks before being analyzed for gallstone formation. Notably, there were visible stones in 100% of PKCβ−/− mice as compared with 10% of WT controls when fed MMLD. Importantly, this increased susceptibility to gallstones in PKCβ−/− mice was accompanied by dramatic increases in both CSIs and hydrophobicity indices (Fig. 7). Comparison of gallstone formation between genotypes is shown in Table 2.

PKCβ−/− Mice Exhibit Lower Expression of Hepatic Cholesterol-7α-hydroxylase (CYP7A1) Gene and Increased Hepatic Phospho-p42/44MAPK Levels—A reduction in biliary bile acids in relation to cholesterol and phospholipids can result from hyposecretion of bile acids. Accordingly, there are many candidate enzymes, transporters, or regulators of hepatic cholesterol metabolism that could theoretically influence the formation of cholesterol gallstones. To understand the molecular mechanism by which PKCβ deficiency promotes lithogenesis, mRNA expression levels of the bile salt export pump ABCB11, the organic cation transporter SLC22A1, and the canalicular cholesterol transporters ABCG5 and ABCG8 were similar in both groups of mice (Fig. 8A), whereas the ATP-binding cassette transporter A1 (ABCA1) showed slightly elevated levels in PKCβ−/− mice as compared with WT mice. Interestingly, PKCβ deficiency down-regulated the classic and alternative pathways of bile salt synthesis by dramatically decreasing hepatic mRNA expression of the CYP7A1 gene (classic pathway) with no change in CYP27A1 mRNA expression (alternative pathway) (Fig. 8A), a finding that is consistent with the increased gallstone formation in PKCβ−/− mice. Additionally, significantly reduced expression of the sterol 12α-hydroxylase (CYP8b1) gene is expected to increase the bile hydrophobicity of PKCβ−/− mice. The reduction in CYP7A1 mRNA expression was not accompanied by any alteration in expression of transcription factors (LXRs, FXR, and SREBP-1c), which are known to regulate its expression (41–43). Interestingly, feeding MMLD caused a greater suppression of both CYP7A1 and CYP8b1 genes in the livers of PKCβ−/− mice as compared with controls (Fig. 8B). We next asked whether the decreased

### Table 2

| Genotype | Diet   | Gallstones |
|----------|--------|------------|
| WT       | MMLD   | 10% (1/10) |
| PKCβ−/−  | MMLD   | 100% (10/10) |

**FIGURE 7.** Biliary lipid secretion rates, cholesterol saturation indices, bile acid species distribution, and hydrophobicity indices in mice fed MMLD for 4 weeks. A and B, WT and PKCβ−/− (n = 6/genotype) mice were fed MMLD containing milk fat for 4 weeks. A total of 10 mice/genotype were analyzed for gallstone formation, and a subset (6/genotype) was used to determine lipid secretion rates and bile acid species. C and D, CSIs and hydrophobicity indices were calculated as described earlier (32, 33). Each value represents the mean ± S.D. (n = 5–10 mice/group). *, p < 0.05.
CYP7A1 mRNA expression was accompanied by a corresponding decrease in protein expression and found decreased microsomal CYP7A1 protein in PKCβ−/− livers as compared with WT mice fed PLD (Fig. 8C). Western blot analysis of CYP7A1 protein expression in liver microsomes prepared as described from chow or PLD-fed animals (n = 4 mice of each genotype). Eighty micrograms of protein was subjected to SDS-PAGE and Western blotting with anti-CYP7A1 IgG and quantitated by imaging with Bip used as a loading control (results not included). Each value represents the mean ± S.D. (n = 5–10 mice/group). *, p < 0.05; **, p < 0.01.

DISCUSSION

The present study defines, for the first time, a component of the hepatic signaling pathway that is required for adaptation to lithogenic diet feeding and implicates PKCβ as a critical kinase in mediating the metabolic response to this diet. Our observation that PKCβ deficiency stimulates diet-induced gallstone formation provides direct evidence that this signaling kinase is important in maintaining cholesterol and bile acid homeostasis. This makes PKCβ an excellent positional candidate for the

FIGURE 8. PKCβ-dependent modulation of expression of CYP7A1 and CYP8b1 genes. A, the expression levels of each gene in WT and PKCβ−/− mice fed PLD for 8 weeks, which were normalized to the expression level in WT mice (n = 5 mice/group). Levels for each gene in WT mice were assigned a value of 1. B, expression levels of indicated genes in the livers of MMLD-fed WT and PKCβ−/− mice. C, Western blot analysis of CYP7A1 protein expression in liver microsomes prepared as described from chow or PLD-fed animals (n = 4 mice of each genotype). Eighty micrograms of protein was subjected to SDS-PAGE and Western blotting with anti-CYP7A1 IgG and quantitated by imaging with Bip used as a loading control (results not included). Each value represents the mean ± S.D. (n = 5–10 mice/group). *, p < 0.05; **, p < 0.01.

FIGURE 9. Increased hepatic phospho-p42/44MAPK abundance in PKCβ−/− mice as compared with WT mice fed PLD. Equal amounts of the whole liver lysates from WT and PKCβ−/− mice fed chow or PLD for 8 weeks were separated by SDS-PAGE, and phosphorylation levels of p42/44MAPK, MEK-1/2, and p38MAPK were analyzed by Western blotting using phospho-specific or phosphorylation-independent p42/44MAPK antibodies following transfer of total proteins onto nitrocellulose. The relative phosphorylation levels were determined by densitometry and are shown in the right panel. Phosphorylation levels for WT were assigned a value of 100% as shown in the graph. Each value represents the mean ± S.D. (n = 5–10 mice/group). *, p < 0.05.
Lith22 gene due to its close proximity within the locus near D7Mit12 on mouse chromosome 7.

Although clinical studies have suggested that obesity is an important risk factor for gallstone disease, little is known regarding the precise metabolic pathways through which obesity contributes to gallstone formation (48). Previous animal studies showed that some but by no means all murine models of obesity are associated with increased gallstone susceptibility. Indeed, prior studies in L-Fabp−/− mice have demonstrated protection against high fat diet-induced obesity in the setting of a strikingly increased susceptibility to lithogenic diet-induced gallstone formation (33). These paradoxical findings suggest that obesity itself is not a gallstone risk but rather that specific alterations in key pathophysiologic pathways leading to obesity may in turn augment susceptibility to both diseases (49). Consistent with these results, our findings demonstrate that protection against high fat diet-induced obesity in PKCβ−/− mice is not accompanied by protection against gallstone formation. On the contrary, PKCβ−/− mice lost more weight when consuming the lithogenic diet and exhibited lower hepatic TG content than did WT controls, suggesting that increased gallstone susceptibility observed in PKCβ−/− mice cannot be attributed to augmented obesity, steatosis, or impaired glucose intolerance. The changes observed in hepatic cholesterol metabolism in lithogenic diet-fed PKCβ−/− mice, by contrast, suggest a plausible mechanism for the increased susceptibility to gallstone formation. In particular, we observed a 50% increase in hepatic cholesterol content, coupled with higher plasma cholesterol levels in lithogenic diet-fed PKCβ−/− mice.

The liver eliminates cholesterol from the body via bile as unmodified cholesterol and through conversion of cholesterol into bile salts (50). This second route represents a major pathway for cholesterol elimination, accounting for approximately half of daily excretion. CYP7A1 catalyzes the first and rate-limiting step in the major classic pathway of bile acid biosynthesis and plays a crucial role in cholesterol balance. In the present study, we found significantly reduced hepatic CYP7A1 expression, coupled with decreased biliary bile salt concentrations, in PKCβ−/− mice as compared with WT mice. The reduction in CYP7A1 gene expression in PKCβ−/− mice is likely responsible for increased susceptibility to gallstone formation as compared with WT mice. Human CYP7A1 deficiency is known to promote premature gallstone formation (51, 52), whereas CYP7A1 transgenic mice provide resistance to gallstone formation (53). Several human studies also suggest that an A to C nucleotide substitution in position −204 of the CYP7A1 promoter is associated with variation in plasma LDL cholesterol concentrations (54, 55). These findings led us to propose that a change in hepatic CYP7A1 expression in PKCβ−/− mice is a critical determinant of gallstone formation in vivo and suggest a plausible mechanism for their increased susceptibility.

Among the most intriguing questions posed by this study is the mechanism whereby CYP7A1 expression is modulated in the setting of PKCβ deletion. The control of CYP7A1 level is known to occur primarily at the transcriptional level and is modulated by the relative ratio of cholesterol to bile acids in the liver. The transcriptional regulation is highly complex and involves a multitude of recognized mechanisms and, most likely, many still elusive regulatory mechanisms (56–58). In rodents, CYP7A1 transcription is up-regulated by sterols through an LXRα-dependent pathway. Conversely, the CYP7A1 gene is down-regulated by FXR through a feedback loop involving both small heterodimer protein (SHP)-dependent and SHP-independent pathways. The results presented here argue strongly in favor of a mechanistic link between PKCβ deficiency and CYP7A1 suppression. The finding that mRNA levels were decreased in PKCβ−/− mice suggests that a transcriptional mechanism is involved. There are many potential mechanisms by which PKCβ either indirectly or directly can affect hepatic CYP7A1 transcription. First, p42/44MAPK activation has been linked with CYP7A1 suppression by both SHP-dependent and SHP-independent pathways (44, 45). One such study has shown that p42/44MAPK-induced phosphorylation of hepatic SHP at serine 26 stabilizes this protein by inhibiting ubiquitin-proteasomal degradation (45). However, lack of a significant change in hepatic SHP content between genotypes argues against involvement of SHP-dependent pathways in CYP7A1 repression.4 Alternately, PKCβ directly phosphorylates liver nuclear receptors (LXR, FXR, and PPARα) and/or the transcription factor SREBP-1c and suppresses CYP7A1 transcription, which is in line with earlier reports showing the ability of PKCs to phosphorylate and modify the above transcription factors (59–63). The absence of changes in LXR (ACBG5/8) or FXR (ACBG11) target genes argues against a mechanism involving these transcriptional regulators. PKCβ has also been shown to regulate a molecular switch between transactivation and transrepression activity of the PPARα (61); loss of PKCβ-mediated phosphorylation is associated with a loss of function for ligand-induced transcriptional activity and a gain of function for transrepression activity. Given the role of PPARα in CYP7A1 repression (65), loss of PPARα phosphorylation in PKCβ−/− mice is expected to cause greater suppression of CYP7A1 expression as compared with WT mice. Furthermore, fat-derived diacylglycerol, which is a strong PKCβ activator, may provide the possible link between PKCβ and modulation of PPARα function and thus may reconcile studies that have shown dietary fat requirement for cholesterol-dependent induction of CYP7A1 expression (66, 67). Future studies will focus on underlying molecular mechanisms linking PKCβ deficiency with CYP7A1 suppression.

Another consideration is whether alteration in gallbladder contractility, based upon the differences observed in gallbladder volumes in the two genotypes, contributes to the increased sensitivity of PKCβ−/− mice to gallstones. Human studies have shown that formation of cholesterol-supersaturated bile in patients with gallstones is causally related to their decreased gallbladder contractility (68, 69). Furthermore, it has been shown in cholesterol-fed animals that the decrease in gallbladder contractility is related to the impairment of cholecystokinin signaling-induced biliary alterations (70). These changes favor cholesterol crystal formation and may pathogenetically

4 W. Huang, R. R. Bansode, Y. Xie, L. Rowland, M. Mehta, N. O. Davidson, and K. D. Mehta, unpublished results.
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contribute to gallstone formation. Cholecystokinin-induced gallbladder contraction via its receptor is mediated by the activation of G-proteins and subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol. Ultimately, these events lead to increased smooth muscle contraction through the PKC, p42/44MAPK, and p38MAPK pathways (71–73). Thus, it is tempting to speculate that enlarged gallbladder size and reduced p38MAPK activation in PKCβ−/− mice may reflect reduced gallbladder emptying due to a decrease in cholecystokinin signaling. Future studies will focus on the potential alteration in the cholecystokinin-dependent signaling network and its physiological relevance to increased lithogenesis in these mice.

Dietary fats are known to modify cholesterol metabolism, thereby affecting the whole body cholesterol homeostasis. The role of dietary fat as an etiological factor for cholesterol gallstone disease has received considerable attention but remains unresolved (74, 75). Epidemiological studies have suggested that cholesterol gallstone disease development is positively correlated with saturated fat intake and negatively related to monounsaturated fat intake (76, 77). Subsequent studies in experimental animal models have, in general, supported this suggestion, but individual studies have shown variable responses to diet-induced cholesterol gallstone disease (78–80). A significant difference in time required for gallstone formation was observed in PKCβ−/− mice despite the fact that both diets contained 1% cholesterol, 0.5% cholate, and similar fat content. The time required for gallstone formation was longest in PKCβ−/− mice consuming cocoa butter diet, whereas mice consuming saturated fat of animal origin showed increased severity of gallstone risk with gallstones visible after 3–4 weeks. Results presented support the hypothesis that risk of diet-induced cholesterol gallstone disease is not only influenced by the degree of fatty acid unsaturation but also by the fatty acid composition.

Finally, we also considered the possibility that alterations in adipokine levels might also play a role in this phenotype (81). Previous studies have demonstrated that adipokines are indeed important in gallstone formation. For instance, increased serum leptin has been shown to enhance biliary cholesterol concentration, resulting in subsequent bile supersaturation with cholesterol and hence an increased risk of gallstones (82). Data from leptin-deficient and leptin-resistant mice have additionally suggested that leptin may decrease gallbladder activity (83) and shorten the formation time of cholesterol crystals (84). In contrast, a lower adiponectin level has been linked with gallstone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85). In the present study, we found a reduction in leptin concentration in stone disease, obesity, and insulin resistance (85).

In summary, we provide compelling evidence that PKCβ deficiency can disrupt the physiological balance between pro- and antilithogenic forces in favor of stone formation, at least in part via repression of CYP7A1 expression. An increased supersaturation of bile with cholesterol as a result of decreased bile salts appears particularly relevant, although the contributions of changes in gallbladder motility or enterohepatic cycling remain to be explored. Our findings suggest that PKCβ deficiency, either quantitative or qualitative, may contribute to alterations in diet-induced gallstone formation. A range of pathophysiological conditions can affect PKCβ expression or activity in humans (64, 86). It will be of particular interest and importance to define whether alterations in PKCβ activity contribute to the pathogenesis of gallstone formation under these disease conditions.

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