Restoration of gallstone susceptibility by leptin in C57BL/6J *ob/ob* mice

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Abstract The absence of leptin due to the *ob* mutation leads to obesity and confers resistance to diet-induced cholesterol gallstone formation in otherwise susceptible C57BL/6J mice. To investigate contributions of obesity and leptin to gallstone susceptibility, C57BL/6J *ob/ob* mice were treated daily with i.p. saline or recombinant murine leptin at low (1 μg/g bw) or high (10 μg/g bw) doses and were pair-fed a lithogenic diet (15% dairy fat, 1.25% cholesterol, 0.5% cholic acid). Weight loss in *ob/ob* mice increased in proportion to leptin dose, indicating that the lithogenic diet did not impair leptin sensitivity. In a dose-dependent manner, leptin promoted cholesterol crystallization and gallstone formation, which did not occur in saline-treated mice. Notwithstanding, leptin decreased biliary lipid secretion rates without enriching cholesterol in bile. Leptin did not affect bile salt hydrophobicity, but did increase the biliary content of the most abundant molecular species of phosphatidylcholine, 16:0–18:2. Treatment with leptin down-regulated 3-hydroxy-3-methylglutaryl CoA reductase and prevented cholesterol from accumulating in liver. Consistent with increased hepatic clearance, leptin decreased plasma HDL cholesterol concentrations. This was accommodated in liver without up-regulation of cholesterol 7α-hydroxylase or Acat. These data suggest that despite the lithogenic diet, endogenous sources constitute a significant proportion of biliary cholesterol during leptin-induced weight loss. Kinetic factors related to cholesterol nucleation, gallbladder contractility, or mucin secretion may have accounted for leptin-induced gallstone formation.—Hyogo, H., S. Roy, and D. E. Cohen. **Restoration of gallstone susceptibility by leptin in C57BL/6J *ob/ob* mice. *J. Lipid Res.* 2003.** 44: **1232–1240.**

Supplementary key words bile salts • phospholipids • bile • liver • obesity

Human obesity is a polygenic disorder, with numerous linked genes, markers, and chromosomal regions (1). Cholesterol cholelithiasis is one among several common diseases to which obese individuals are particularly predisposed (2, 3). Cholesterol overproduction (4) and hypersecretion into bile (5–7) set the stage for gallstone formation.

Leptin is a cytokine-like hormone that is produced by adipocytes and plays a pivotal role in the regulation of body weight (8). Most obese individuals appear to be resistant to leptin action. As a result, plasma leptin levels vary in proportion to body fat mass (9). Whereas obesity due to mutations in leptin or the leptin receptor is rare, such genetically obese rodents are valuable for delineating the role of leptin in obesity-related disorders in humans, including cholesterol gallstone formation. In this connection, we have reported that obesity in leptin receptor-defective Zucker (*fa/fa*) rats is associated with decreased biliary cholesterol secretion due to uncoupling of cholesterol and phospholipid from bile salt secretion (10). Because of residual responsiveness of the receptor, acute intravenous administration of high-dose leptin (7 μg/g/day) partially restored biliary cholesterol secretion. We have also shown that weight loss in chow-fed C57BL/6J *ob/ob* mice induced by chronic intraperitoneal administration of high-dose leptin (10 μg/g/day) is associated with cholesterol gallstone formation (11). Under these conditions, supersaturation of bile with cholesterol is due to leptin-induced decreases in bile salt hydrophobicity. In separate studies, Goldblatt et al. (12) have observed decreases in gallbladder contractililty in *ob/ob* compared with lean C57BL/6J mice and have speculated that decreased gallbladder motility contributes to cholelithiasis in obesity.

Among inbred strains of laboratory mice, wild-type male C57BL/6J mice are relatively susceptible to cholesterol gallstones when challenged with a high-fat/high-cholesterol lithogenic diet (13). Homozygosity of C57BL/6J mice for mutations in either leptin (*ob*) or the leptin receptor (*db*), however, is associated with marked decreases in susceptibility to diet-induced cholesterol gallstones (14). Considering that C57BL/6J *ob/ob* mice retain the capacity to respond to leptin (15, 16), exogenous administration

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Abbreviations: Cyp7A1, cholesterol 7α-hydroxylase; FPLC, fast protein liquid chromatography.

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of the hormone would be expected to restore gallstone susceptibility.

Treatment of C57BL/6J *ob/ob* mice with leptin at doses in the range of 5–10 μg/g/day for up to 30 days is required to reduce weights to those of wild-type mice (15, 16); however, in the absence of changes in body weight, administration of leptin at low doses (≤1.0 μg/g/day) and for short periods of time (as few as 2 days) is sufficient to reverse abnormalities of HDL metabolism (17) and transcriptional control of cholesterol homeostasis (18). The current study was undertaken to assess whether low-dose leptin is sufficient to restore susceptibility to diet-induced cholesterol cholelithiasis in C57BL/6J *ob/ob* mice, or whether obesity must first be eliminated using high-dose leptin. Although mice treated with leptin at low and high doses developed cholesterol gallstones when challenged with a lithogenic diet, our data suggest that the pathogenic mechanisms are distinct from those in wild-type mice.

MATERIALS AND METHODS

Materials

Recombinant murine leptin was a gift from Amgen (Thousand Oaks, CA). General chemical reagents were obtained from Sigma (St. Louis, MO) and radiochemicals were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO), DuPont NEN (Boston, MA) and Amersham (Clearbrook, IL).

Experimental design

Male 8-week old C57BL/6J mice homozygous for the *ob* mutation were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in a temperature-controlled room with 12:12 h day-night cycles (6 AM to 6 PM light). Upon arrival, mice were allowed to adapt to the environment for 2 weeks on a chow diet (LabDiet 5001, PMI Nutrition International Inc., Brentwood, MO) that contained 4.5% fat and <0.02% cholesterol. At 10 weeks of age, mice were fed a semisynthetic high-fat/high-cholesterol lithogenic diet (TD90221; Harlan Teklad, Madison, WI) composed of 15% total fat, 1.25% cholesterol, and 0.5% cholic acid. Mice (n = 65) were divided into three groups and were treated once daily with equal-volume intraperitoneal (ip) injections of saline or leptin dissolved in saline at low (1 μg/g bw) or high (10 μg/g bw) doses for periods ranging from 0 to 28 days. To achieve isocaloric intake, mice treated with either saline or low-dose leptin were pair-fed with mice treated with high-dose leptin.

Following anesthesia with i.p. injection of 87 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 13 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA), surgery commenced at 9 AM with a midline abdominal incision. The gallbladder was visually inspected for the presence of gallstones, which were confirmed subsequently using light microscopy. Next, the common bile duct was ligated with silk sutures. Bile flow was diverted for collection by inserting a PE-10 polyethylene catheter (Becton Dickinson Primary Care Diagnostics, Becton Dickinson, Sparks, MD) into the gallbladder and securing it with silk sutures. The cannula was externalized, and the abdominal incision was closed. The first ~10 μl containing concentrated gallbladder bile was collected onto a glass microscopy slide for microscopic analysis to determine the presence of cholesterol crystals (19). Thereafter, hepatic bile was collected by gravity into preweighed Eppendorf tubes for 2 h periods. We found that this approach yielded consistent flow rates, with less than ±8% variability. Bile volume was determined gravimetrically, assuming a density of 1 g/ml. During the 2 h collection period, mice were kept anesthetized by repeat administration of ketamine and xylazine at half the original dose. At the end of the experiment, mice were euthanized by cardiac puncture. Livers were immediately excised, rinsed with 0.15 M NaCl to remove blood, weighed, and snap frozen in liquid nitrogen. Tissue samples were stored at −80°C prior to analyses. Blood was anticoagulated with EDTA, and plasma was separated by centrifugation and maintained at 4°C for analysis within 24 h. During surgery and hepatic bile collections, body temperature was maintained at 37°C ± 0.5°C with a heat lamp. These procedures were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Analytical techniques

Lipid analyses. Plasma cholesterol and triglyceride concentrations were determined by enzymatic assays using reagents from Sigma and Boehringer Mannheim/Roche (Indianapolis, IN), respectively. Plasma lipoproteins were fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (10, 11). Plasma cholesterol concentrations contained in VLDL, LDL/HDL1, and HDL particles were calculated as products of plasma total cholesterol concentrations and relative FPLC peak areas of respective lipoprotein fractions (10). Hepatic and adipose tissue contents of triglycerides and total as well as free cholesterol were quantified following organic extraction using enzymatic assays (10, 20).

Biliary cholesterol, phospholipid, and bile salt concentrations and bile salt species were measured as previously described (10). Bile salt hydrophobic index was determined according to Heuman (21). Cholesterol, phospholipid, and bile salt secretion rates (nmol/h) were calculated as products of lipid concentrations and bile flow rates. Biliary bile salt concentrations and compositions were determined by HPLC (10) utilizing glycocholate as an internal standard. Molecular species of phosphatidylcholines in bile were quantified by HPLC (22, 23).

Enzyme activities. Microsomes were prepared from 500 mg of liver by differential ultracentrifugation (24) and stored at −80°C. Microsomal protein concentrations were determined according to the Bradford method (25) using a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and BSA as a standard. Microsomes were used to measure activities of HMG-CoA reductase (EC 1.1.1.3.4), cholesterol 7α-hydroxylase (Cyp7A1), (EC 1.14.13.17) and Acat (E.C. 2.3.1.26) (11).

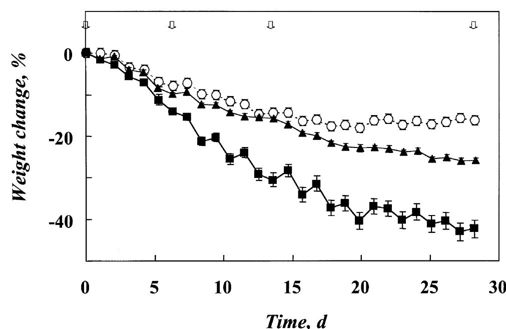

Fig. 1. Influence of leptin on body weight in C57BL/6J *ob/ob* mice fed a lithogenic diet. Mice (n = 51) received either daily intraperitoneal injections of saline (circle, n = 12) or leptin at low (1 μg/g, triangle, n = 12) or high (10 μg/g, square, n = 27) doses. The baseline body weight at day 0 was established using n = 14 mice. Vertical arrows indicate time points at which experiments were performed. Data are mean ± SEM.

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TABLE 1. Lipid compositions of hepatic biles

Day	Concentration				Moles		
	Cholesterol	PL	BS	TL	Cholesterol	PL	BS
	mM			*g/dl*	*%*		
0	0.6 ± 0.1[a]	7.2 ± 0.8	20.6 ± 1.4	1.6 ± 0.1	2.3 ± 0.1	25.0 ± 1.6	72.7 ± 1.5
Saline							
7	1.2 ± 0.1[b]	11.3 ± 0.7[b]	31.7 ± 1.5[b]	2.5 ± 0.2[b]	4.4 ± 0.1[b]	25.3 ± 0.4	70.4 ± 0.5
14	2.1 ± 0.1[b]	14.9 ± 0.2[b]	39.7 ± 2.0[b]	3.3 ± 0.1[b]	3.8 ± 0.3[b]	26.0 ± 0.7	70.2 ± 0.8
28	3.4 ± 0.2[b]	17.8 ± 0.4[b]	44.0 ± 1.9[b]	3.7 ± 0.2[b]	5.1 ± 0.2[b]	27.4 ± 0.6	67.4 ± 0.4[a]
Leptin (1 μg/g)							
7	1.8 ± 0.1[b,c]	14.6 ± 0.8[b,c]	33.9 ± 1.4[b]	2.9 ± 0.1[b]	4.2 ± 0.1[b]	28.9 ± 1.8[c]	66.9 ± 1.8[b,c]
14	2.2 ± 0.6[b]	13.2 ± 1.1[b]	25.9 ± 4.5[c]	2.4 ± 0.4[c]	5.1 ± 0.7[b]	33.0 ± 2.4[c]	61.9 ± 2.0[b,c]
28	1.7 ± 0.1[b,c]	14.5 ± 1.2[b]	38.1 ± 2.7[b]	3.1 ± 0.2[b]	4.5 ± 0.5[b]	26.0 ± 1.2	69.4 ± 1.6
Leptin (10 μg/g)							
7	1.2 ± 0.2[b]	11.9 ± 0.3[b]	27.3 ± 2.9	2.4 ± 0.2[b]	4.4 ± 0.3[b]	30.3 ± 0.9[b,c]	65.4 ± 1.0[b,c]
14	1.3 ± 0.1[b,c]	12.1 ± 0.4[b,c]	25.5 ± 3.2[c]	2.2 ± 0.2[b,c]	3.5 ± 0.5[b]	31.8 ± 3.1[b]	64.6 ± 3.4[b,c]
28	2.3 ± 0.1[b,c]	12.7 ± 0.6[b,c]	37.5 ± 3.0[b]	2.9 ± 0.2[b]	4.6 ± 0.4[b]	24.5 ± 2.30	70.9 ± 2.7

BS, bile salt; PL, phospholipid; TL, total lipid concentration.
[a] Values are mean ± SEM and were determined from n = 5 mice per group at each time point.
[b] $P < 0.05$, compared with baseline.
[c] $P < 0.05$, compared with saline treatment.
[d] $P < 0.05$, compared with leptin (1 μg/g) treatment.

Statistical methods. Data are expressed as means ± SEM. Statistical significance of the differences between the means of the experimental groups was tested by the Student's *t*-test. A difference was considered statistically significant for a two-tailed $P < 0.05$.

RESULTS

Injection of leptin resulted in dose- and time-dependent reductions in intake of the lithogenic diet by 70–80% in the first week and 40–60% thereafter. As a consequence of pair feeding, saline- and leptin-treated mice lost weight (**Fig. 1**). Weight loss was most rapid in the first 14 days and leveled off by 28 days. Leptin treatment increased weight loss in proportion to the administered dose. Final body weights (g, mean ± SEM) were 46.4 ± 0.3, 40.3 ± 0.7, and 31.6 ± 1.1 for mice treated with saline, low-dose, and high-dose leptin, respectively.

As indicated in Fig. 1, the physical states of biliary lipids were examined by light microscopy at baseline and at 7, 14, and 28 days (n = 4–14 mice/group). At baseline and during saline treatment, neither gallstones nor cholesterol crystals were observed. In mice treated with low-dose leptin, both gallstones and cholesterol monohydrate crystals were observed in all mice at 28 days, but in no mice prior to this time. In mice treated with high-dose leptin, cholesterol monohydrate crystals were observed in all mice at 7, 14, and 28 days. Gallstones were present in 5/9 mice at 14 days and in all mice at 28 days.

Table 1 presents the biliary lipid compositions of hepatic biles of biliary lipids from saline- and leptin-injected mice.[3] Compared with baseline (day 0), absolute (mM) and relative (mol%) concentrations of biliary cholesterol were increased at all time points on the lithogenic diet. When compared with saline treatment, low-dose leptin increased biliary cholesterol concentrations at 7 days, after which values decreased to less than saline controls at 28 days. In mice treated with high-dose leptin, absolute cholesterol concentrations were unchanged at 7 days, and then decreased progressively (compared with saline treatment). Leptin at low or high doses had no effect on relative cholesterol concentrations. Similar trends were observed for absolute concentrations of biliary phospholipids and bile salts as were observed for cholesterol. The main exception was that bile salt concentrations increased in leptin-treated animals after 28 days on the lithogenic diet to the level observed in saline-treated mice. Low- and high-dose leptin treatment increased the relative phospholipid concentration at 7 and 14 days compared with baseline and saline treatment. Thereafter, values returned to baseline. In contrast, relative bile salt concentrations decreased at 7 and 14 days, before returning to baseline at 28 days. Considering that bile salts promote biliary secretion of phospholipids and cholesterol by molecular coupling that occurs at the canalicular plasma membrane (28), the observed changes in biliary lipid compositions may reflect leptin-induced changes in the composition of the canalicular membrane. **Figure 2** presents biliary lipid secretion rates without normalization to body weight (5, 11). The lithogenic diet increased biliary secretion rates of cholesterol (Fig. 2A), phospholipids (Fig. 2B), and bile salts (Fig. 2C) in saline-treated mice. These changes were eliminated by increasing doses of leptin.

The effects of leptin on biliary bile salt and phosphatidylcholine molecular species are shown in **Table 2**. Consistent with feeding a cholate-containing lithogenic diet, biles became enriched in taurocholate and taurodeoxycholate at the expense of the other bile salt species. This resulted in increases in the hydrophobic index, a calcu-

[3] Cholesterol saturation indices (CSI) of biles are not included in Table 1. This is because accurate calculations of CSI require that the composition of biliary bile salts be similar to human bile (26, 27). This criterion is not met by the current experimental conditions.

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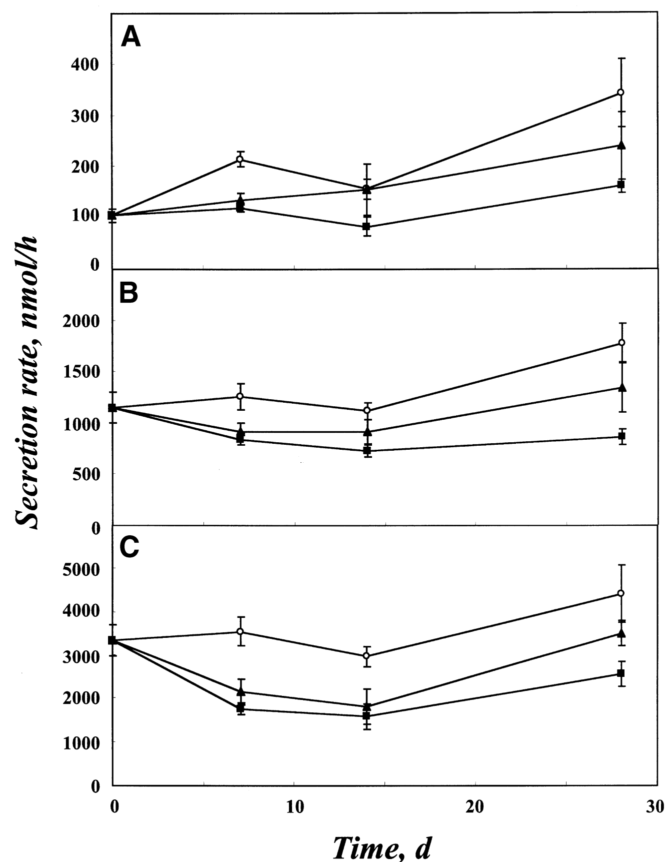

Fig. 2. Biliary secretion rates of (A) cholesterol (B) phospholipid and (C) bile salts for *ob/ob* mice treated with saline (circle, n = 12) or leptin at low (1 μg/g/day, triangle, n = 12) or high (10 μg/g/day, square, n = 27) doses. Baseline biliary lipid secretion rates at day 0 were established using n = 14 mice. Secretion rates were calculated as products of bile flow rates and biliary lipid concentrations, and are plotted as functions of time on the lithogenic diet. Data symbols represent mean ± SEM.

lated concentration-weighted average of the hydrophobicities of individual bile salts (21). Biliary bile salts became more hydrophobic in a time-dependent manner. Whereas there were no significant changes in hydrophobic index between groups, it did appear that enrichment of bile with taurodeoxycholate was accelerated by leptin treatment. In addition, leptin significantly altered the composition of biliary phosphatidylcholines. High-dose leptin treatment increased the proportions of 16:1–18:2 and 16:0–18:2 and decreased the proportions of 16:1–20:4, 16:0–20:4, 16:0–18:1/18:0–20:4, and 18:0–18:2 phosphatidylcholines.

Figure 3 displays representative elution profiles for plasma lipoproteins from mice after 4 weeks of treatment with saline or high-dose leptin. The proportion of VLDL cholesterol increased in both groups, but was not influenced by leptin. The proportion of LDL/HDL1 cholesterol also increased in both groups of mice, but this was more pronounced in mice administered leptin. The proportion of HDL cholesterol was decreased to a greater degree in leptin-treated mice. Although not displayed, for clarity, lipoprotein profiles obtained for mice treated with low-dose leptin were intermediate.

Figure 4 summarizes the influence of leptin on plasma lipid concentrations. During the 28 day feeding period, plasma total cholesterol concentrations increased to similar extents in leptin- and saline-treated mice (Fig. 4A). HDL cholesterol decreased in both groups of mice (Fig. 4B); however, there were substantially greater reductions in leptin-treated animals. LDL/HDL1 cholesterol concentrations increased to a greater extent in leptin-treated mice at 28 days (Fig. 4C). VLDL cholesterol concentrations, which increased linearly as a function of time, were not influenced by leptin treatment (Fig. 4D). Plasma triglycerides remained essentially unchanged during the course of the experiment (Fig. 4E).

Figure 5 illustrates the influence of leptin on hepatic lipids and enzyme activities. As was the case in Figs. 3 and 4, values for mice treated with low-dose leptin are not shown for clarity, but were intermediate between saline and high-dose leptin. As depicted in Fig. 5A, hepatic concentrations (mg/g liver) and contents (mg/liver) of cholesterol increased sharply in saline-treated mice.[4] These changes were markedly attenuated by leptin treatment, which prevented increases in cholesterol concentrations and contents at 14 days and allowed for only slight increases at 28 days. Leptin did not influence the free cholesterol-esterified cholesterol ratio (unpublished information). Contents and concentrations of triglycerides in liver decreased progressively during the course of the experiment in both saline- and leptin-treated mice (Fig. 5B). Whereas the concentrations of hepatic triglycerides were unaffected by leptin treatment, triglyceride contents were decreased at 28 days.

Figure 5C displays hepatic activities of HMG-CoA reductase, Cyp7A1, and Acat. Measurements were made following 14 days on the lithogenic diet, at which point leptin had suppressed the lithogenic diet-induced increases in hepatic cholesterol contents and concentrations (Fig. 5A). HMG-CoA reductase activity was decreased in both

[4] Although livers were rinsed thoroughly with saline in these experiments, portal veins were not flushed. As a result, blood may not have been eliminated completely from the liver prior to measurements of liver lipids. In addition, intrahepatic bile might still have been present in the liver. Because blood and bile contain cholesterol, the measurements of tissue cholesterol in Fig. 5A may have been influenced by the cholesterol concentrations in these fluids. We therefore estimated the potential contributions of cholesterol from blood and bile within the liver. Based on measurements performed in rats, the liver contains ∼0.2 ml of blood/g (29), and ∼3 μl of bile/g (30). Assuming a plasma lipoprotein cholesterol concentration of 150 mg/dl (Fig. 4A), and a density of liver tissue, of blood, and of bile equal to 1 g/ml, then each gram of liver tissue includes 0.3 mg that is attributable to plasma lipoprotein cholesterol. Membranes of erythrocytes also contain cholesterol, which amounts to a concentration of ∼100 mg/dl in mouse blood (31). Therefore, the potential contribution from erythrocytes to hepatic cholesterol is 0.2 mg/g of tissue. Because the concentration of cholesterol in hepatic bile ranges up to 130 mg/dl (Table 1), there is at most 0.004 mg per gram of liver of biliary cholesterol, an amount that is insignificant compared with the contribution from blood. Therefore, the estimated amount of cholesterol per gram of liver that may actually have derived from blood and bile is 0.5 mg. Considering that the lowest hepatic cholesterol concentration was 8.4 mg/g at baseline in this study (Fig. 5A), the maximum estimated contribution of cholesterol from blood and bile was ∼6%.

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TABLE 2. Bile salt and phosphatidylcholine molecular species in hepatic biles

	Baseline	Saline			Leptin			Leptin		
Day	0	7	14	28	7	14	28	7	14	28
						1 μg/g			10 μg/g	
BS species										
TMC	28.2 ± 1.3[a]	14.0 ± 1.9[b]	9.9 ± 1.4[b]	3.9 ± 0.4[b]	9.6 ± 0.2[b]	12.0 ± 1.2[b]	3.2 ± 0.2[b]	11.3 ± 1.0[b]	18.2 ± 0.8[b,c]	6.2 ± 0.7[b,c,d]
TUDC	2.2 ± 0.2	0.8 ± 0.2[b]	0.4 ± 0.1[b]	0.7 ± 0.1[b]	0.7 ± 0.1[b]	0.7 ± 0.1[b]	0.3 ± 0.1[b]	0.7 ± 0.2[b]	0.7 ± 0.2[b]	0.2 ± 0.1[b]
TC	56.4 ± 2.9	74.1 ± 4.0[b]	73.5 ± 3.5[b]	73.5 ± 1.7[b]	74.5 ± 2.0[b]	66.7 ± 1.9[b]	63.3 ± 2.0[b]	72.0 ± 0.8[b]	61.1 ± 2.3[b,c]	68.7 ± 2.5[b]
TCDC	3.9 ± 0.5	0.9 ± 0.2[b]	0.5 ± 0.1[b]	3.7 ± 1.1	1.0 ± 0.1[b]	2.3 ± 1.5	8.0 ± 0.5	1.1 ± 0.1[b]	1.9 ± 0.2[b,c]	3.4 ± 0.6[d]
TDC	6.2 ± 1.5	3.1 ± 0.6	4.8 ± 0.9	12.5 ± 0.6[b]	5.7 ± 0.9	11.4 ± 2.6	15.3 ± 2.1[b]	6.4 ± 0.2[c]	11.1 ± 2.8[c]	13.8 ± 2.0[b]
HI	−0.18 ± 0.01	−0.09 ± 0.01[b]	−0.05 ± 0.01[b]	0.06 ± 0.01[b]	−0.04 ± 0.01[b]	−0.02 ± 0.03[b]	0.10 ± 0.01[b]	−0.05 ± 0.01[b]	−0.07 ± 0.02[b]	0.05 ± 0.01[b]
PC species										
16:1–16:1	0.5 ± 0.1	0.6 ± 0.0	1.0 ± 0.1[b,d]	1.4 ± 0.2[b]	0.7 ± 0.1	0.8 ± 0.1[b,d]	1.0 ± 0.1[b]	0.7 ± 0.1	0.9 ± 0.1[b]	0.5 ± 0.1[c]
16:1–20:4	4.4 ± 0.3	5.4 ± 0.2[b]	6.3 ± 0.3	6.0 ± 0.3[b]	4.6 ± 0.3	5.1 ± 0.3[c]	5.8 ± 0.2[b]	4.3 ± 0.1[c]	4.2 ± 0.1[c,d]	3.8 ± 0.1[c,d]
16:1–18:2	1.8 ± 0.2	2.5 ± 0.1	4.2 ± 0.1[b]	3.7 ± 0.2[b,d]	2.4 ± 0.2	3.9 ± 0.1[b]	4.7 ± 0.1[b,c]	2.9 ± 0.2[b]	4.6 ± 0.1[b,c,d]	4.2 ± 0.1[b,d]
16:0–22:6	5.4 ± 0.4	6.6 ± 0.4	6.0 ± 0.2	4.7 ± 0.3	6.8 ± 0.3	6.2 ± 0.9	5.3 ± 0.10	6.3 ± 0.5	6.1 ± 0.6	5.0 ± 0.1
16:0–20:4	10.8 ± 0.5	12.0 ± 0.4	8.7 ± 0.6	10.2 ± 0.2	13.1 ± 0.3	9.1 ± 0.3	9.2 ± 0.30	10.2 ± 0.4[c,d]	8.3 ± 0.7[c,d]	9.5 ± 0.1
16:0–18:2	45.7 ± 2.7	47.8 ± 0.8	53.3 ± 1.4	43.0 ± 1.7	47.6 ± 0.4	52.8 ± 2.5	46.8 ± 0.9	52.8 ± 1.2[c,d]	56.8 ± 1.2	51.0 ± 0.2[c,d]
16:0–18:1/18:0–20:4	22.4 ± 2.0	16.5 ± 0.4[b]	12.9 ± 0.6[b]	22.2 ± 1.3	16.3 ± 0.7[b]	14.8 ± 1.5[b]	19.8 ± 0.6	13.4 ± 0.5[b,c,d]	11.8 ± 0.6[b]	22.0 ± 0.3
18:0–18:2	5.5 ± 0.7	4.0 ± 0.4	3.85 ± 0.2	3.6 ± 0.1[b,d]	3.7 ± 0.2	3.4 ± 0.3	2.7 ± 0.1[b,c]	4.6 ± 0.2[d]	4.1 ± 0.2	3.1 ± 0.1[b,c]
18:0–18:1	nd	0.4 ± 0.1	0.23 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.5 ± 0.1

HI, hydrophobic index; nd, not detected; PC, phosphatidylcholine; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TMC, tauromuricholate; TUDC, tauroursodeoxycholate.

[a] Values are mean ± SEM and were determined from n = 5 mice per group at each time point.

[b] $P < 0.05$, compared with baseline.

[c] $P < 0.05$, compared with saline treatment.

[d] $P < 0.05$, compared with leptin (1 μg/g) treatment.

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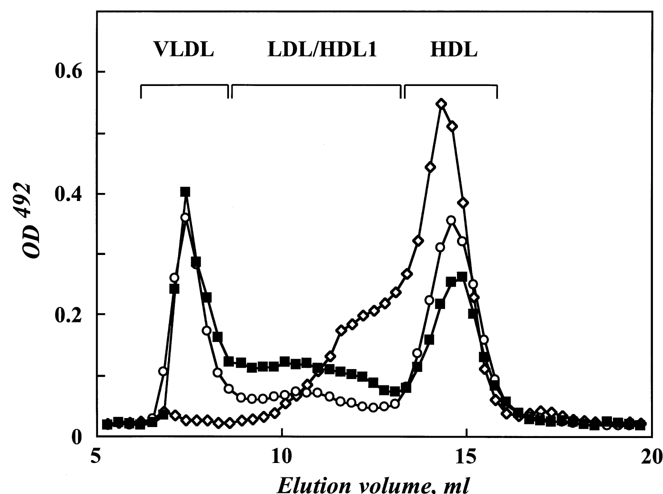

Fig. 3. Influence of leptin on plasma lipoproteins in *ob/ob* mice. Lipoproteins in equal volumes of plasma from mice prior to (diamond, n = 14) and after feeding a lithogenic diet for 28 days during treatment with saline (circle, n = 12) or high-dose leptin (10 μg/g) (square, n = 12) were pooled and 200 μl was fractionated by FPLC. Cholesterol concentrations in individual fractions were determined enzymatically and represented as optical density measured at 492 nm. Distributions of VLDL, LDL/HDL1, and HDL among eluted fractions are indicated.

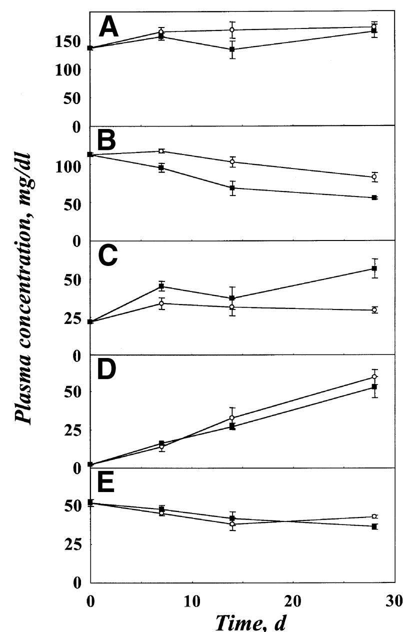

Fig. 4. Plasma lipid concentrations in *ob/ob* mice fed a lithogenic diet and treated with saline (circle) or high-dose leptin (10 μg/g) (square). Plasma concentrations of (A) total cholesterol, (B) HDL cholesterol, (C) LDL/HDL1 cholesterol, (D) VLDL cholesterol, and (E) total triglycerides are plotted as functions of days on the lithogenic diet. Lipoprotein cholesterol concentrations were calculated as products of total cholesterol concentration and fractions of cholesterol contained in respective lipoprotein particles, which was determined by FPLC analysis as shown in Fig. 3. Data symbols represent mean ± SEM.

saline- and leptin-treated mice, but the decrease was much more pronounced with leptin. Activities of both Cyp7A1 and Acat were increased in saline-treated mice compared with baseline. Leptin treatment eliminated diet-induced increases in Cyp7A1 activity and markedly attenuated the increase in activity of Acat.

DISCUSSION

Whereas C57BL/6J mice are genetically susceptible to diet-induced cholesterol cholelithiasis, the *ob* mutation confers resistance in mice of this strain. This study was designed to examine the interactions between obesity, leptin, and a lithogenic diet in determining the susceptibility to cholesterol gallstones in inbred mice. We observed that chronic leptin administration at low and high doses restored susceptibility to cholesterol gallstones.

Despite the high-fat/high-cholesterol-containing lithogenic diet, pair-fed mice treated with leptin or saline displayed similar patterns of weight loss as have been reported for chow-fed mice (11, 15, 16). Therefore, the composition of the diet did not substantially impair the sensitivity of *ob/ob* mice to leptin administration. In our previous study (11), chow-fed *ob/ob* mice treated with high-dose leptin lost 30% of their body weight over a 14 day period. This was associated with cholesterol crystals and gallstones in 44% and 11% of mice, respectively. The same rate of weight loss due to high-dose leptin (i.e., 30% of body weight in 14 days) in *ob/ob* mice fed the lithogenic diet (Fig. 1) resulted in cholesterol crystals and gallstones in 100% and 55% of mice, respectively. A 25% reduction in body weight over a 28 day period was sufficient to yield cholesterol crystals and gallstones in gallbladder biles of all lithogenic diet-fed mice treated with low-dose leptin. Whereas low-dose leptin was not utilized in our prior study (11), a similar rate of weight loss occurred in chow-fed mice treated with saline as was observed here for lithogenic diet-fed mice treated with low-dose leptin. Nevertheless, neither cholesterol crystals nor stones were observed in any chow-fed mouse (11). These findings indicate that, whereas leptin replacement is required for cholesterol cholelithiasis in *ob/ob* mice, the lithogenic diet accelerates this process.

The biochemical and physical-chemical events leading to diet-induced cholesterol gallstone formation in C57BL/6J mice and the closely related C57L/J strain have been characterized extensively (32–34). When challenged with a lithogenic diet, hypersecretion of cholesterol compared with phospholipids and bile salts leads to supersaturation of gallbladder bile with cholesterol. Upon feeding the lithogenic diet, the bile salt pool becomes enriched in cholate, deoxycholate, and chenodeoxycholate (35). These hydrophobic bile salts are largely responsible for hypersecretion of biliary cholesterol (36). As predicted by equilibrium-phase relationships of bile that is both supersaturated with cholesterol and contains principally hydrophobic bile salts, cholesterol crystallizes within the gallbladder first as anhydrous cholesterol in a variety of crystal forms (19). With time, cholesterol molecules become hydrated and trans-

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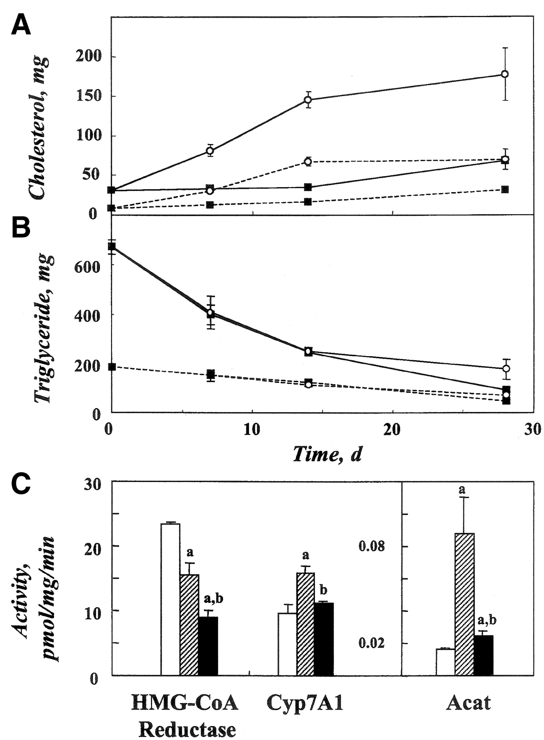

Fig. 5. Leptin regulates lipids and enzyme activities in liver. Hepatic concentrations (mg/g liver, dashed lines) and contents (mg/liver, solid lines) of (A) cholesterol and (B) triglyceride from *ob/ob* mice treated with saline (circle, n = 12) or high-dose leptin (10 μg/g) (square, n = 12) are plotted as functions of time on the lithogenic diet. C: Activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and *Acat* in *ob/ob* mice were determined at baseline (open bars, n = 5) and following 14 days of treatment with saline (hatched bars, n = 5) or high-dose leptin (10 μg/g) (solid bars, n = 5). Data are means ± SEM. [a]$P < 0.05$ versus baseline; [b]$P < 0.05$ versus saline.

form into plate-like cholesterol monohydrate crystals, which comprise the building blocks of macroscopic cholesterol gallstones (2). By contrast, in leptin-treated *ob/ob* mice fed a regular chow diet, cholelithiasis followed crystallization of cholesterol as monohydrate crystals without first forming anhydrous cholesterol intermediate forms (11). Supersaturation of bile with cholesterol in these animals was the result of marked leptin-induced decreases in bile salt hydrophobicity that were not accompanied by reductions in biliary cholesterol secretion. Under these conditions, model bile systems predict that cholesterol should indeed crystallize directly into its monohydrate form (19). As was the case in chow-fed *ob/ob* mice, we observed cholesterol monohydrate crystals exclusively in gallbladder biles of leptin-treated mice that were challenged with the lithogenic diet. This occurred despite the fact that the cholate-containing diet progressively increased the hydrophobic index of biliary bile salts (Table 2) to similar final values as in gallstone-susceptible C57L/J mice in which cholesterol crystallizes first in anhydrous forms (35). Although leptin did appear to accelerate the accumulation of taurodeoxycholate in bile (Table 2), the lack of anhydrous crystal forms would argue against a mechanistic role in promoting cholesterol gallstone formation (19).

Considering that cholesterol crystals and gallstones formed only in leptin-treated mice, we did not expect to find that leptin treatment decreased the absolute and relative concentrations of cholesterol in bile without substantially affecting bile salts and phospholipids (Table 1). This means that mechanisms of leptin action, other than promoting hypersecretion of biliary cholesterol, must have contributed to gallstone susceptibility in our experiments. Changes in biliary phosphatidylcholine compositions could explain leptin-induced supersaturation of bile, despite lower cholesterol concentrations. Studies in model (22, 37) and native (38) biles have demonstrated that biliary phosphatidylcholine compositions may play an important role in determining cholesterol solubility. In general, cholesterol crystallizes more rapidly in the presence of phosphatidylcholine molecular species that contain more unsaturated acyl chains. Leptin treatment increased the concentration of the most abundant phosphatidylcholine molecular species in bile (i.e., 16:0–18:2) and had varied effects on the other molecular species (Table 2). It is possible that these changes may have been sufficient to explain accelerated crystallization; however, unlike bile salts (19, 26), the effects on cholesterol solubility of phosphatidylcholine mixtures have not been quantified systematically in model systems.

A feature of the current experimental design is that the bile composition and biliary bile salt hydrophobicity were effectively "clamped" by the lithogenic diet. In this setting, the persistence of a lithogenic effect might be interpreted as evidence that leptin's effects on biliary bile salt hydrophobicity and biliary cholesterol saturation in chow-fed mice (11) were not critical for gallstone formation; however, it is important to point out that the addition of the lithogenic diet may have substantially altered the model. In the current study, bile salt hydrophobicity was increased by the lithogenic diet, whereas leptin administration to chow-fed mice markedly decreased bile salt hydrophobicity (11). As a consequence, driving forces for cholesterol crystallization differed, suggesting that gallstone formation in *ob/ob* mice fed the lithogenic diet does not likely reflect an inherent feature of the chow-fed model. Rather, kinetic factors related to cholesterol nucleation, gallbladder contractility, or mucin secretion may have accounted for the higher gallstone prevalence following leptin treatment under the current experimental conditions. In this connection, Goldblatt et al. (12) have demonstrated alterations in gallbladder motility in *ob/ob* mice that are reversed by leptin administration.

In a recent report, Wang and Carey (39) demonstrated that susceptibility to cholesterol gallstone formation in mice is not affected by disruption of the HDL receptor SR-BI, indicating that dietary cholesterol is a critical source of biliary cholesterol during lithogenic diet-induced gallstone formation. This is in contrast to leptin-induced cholesterol cholelithiasis in chow-fed *ob/ob* mice (11). In this model, leptin promoted clearance of endogenous cholesterol that was liberated from peripheral tissues in response to hydrolysis of endogenous triglycerides. Our cur-

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rent data suggest that, even when *ob/ob* mice are fed the lithogenic diet, endogenous cholesterol remains an important source of excess biliary cholesterol in leptin-treated mice. In mice treated with saline, the lithogenic diet results in hepatic cholesterol accumulation. The liver responds by decreasing cholesterol synthesis and increasing esterification, as well as by increasing catabolism to bile salts (Fig. 5C). Consistent with previous observations (10, 11, 18), leptin administration down-regulates cholesterol biosynthesis (i.e., activity of HMG-CoA reductase). The extent of down-regulation of synthesis is sufficient to prevent hepatic cholesterol accumulation (Fig. 5A) and to accommodate leptin-stimulated uptake of HDL cholesterol (Fig. 4B) (17, 40) without the need to up-regulate bile salt synthesis (i.e., Cyp7A1 activity) or cholesterol esterification (i.e., Acat activity) (Fig. 5C). These coordinate responses strongly suggest that HDL-derived cholesterol is secreted preferentially into bile in response to leptin administration.

Our finding that leptin is a critical cofactor for gallstone formation in *ob/ob* mice is in apparent contradiction with the results of a recent, large epidemiological study showing that plasma leptin concentration is no better a predictor of gallbladder disease than is anthropometry (41). These authors concluded that leptin is not a major mediator of the relationship between obesity and gallbladder disease, an assertion consistent with the observation that most obese humans have high leptin levels and are resistant to leptin action (9). Whereas we have demonstrated that leptin administration restores susceptibility to a lithogenic diet in leptin-sensitive *ob/ob* mice, the physiological and physical chemical mechanism(s) of cholesterol gallstone formation differ(s) from genetically susceptible mouse strains (35, 42) and humans (43). The observed changes in plasma and biliary lipids, coordinated responses of hepatic enzymes of cholesterol metabolism, and pattern of cholesterol crystal formation strongly suggest that endogenous cholesterol mobilized during leptin-induced weight loss played a key role in promoting gallstone formation (11). These changes more closely reflect the pathophysiology of biliary lipid secretion that occurs during rapid weight loss (5). In this connection, Mendez-Sanchez and coworkers (44) have demonstrated that, although leptin levels and biliary cholesterol saturation were not correlated in a group of obese subjects, a significant positive correlation emerged following a modest degree of weight loss (44). We speculate that the increased risk of gallstones faced by obese individuals who lose weight rapidly (2) may be attributable to restoration of leptin sensitivity that occurs prior to a decrease in plasma leptin levels.

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