Leptin administration to obese C57BL/6J (ob/ob) mice results in weight loss by reducing body fat. Because adipose tissue is an important storage depot for cholesterol, we explored evidence that leptin-induced weight loss in ob/ob mice was accompanied by transport of cholesterol to the liver and its elimination via bile. Consistent with mobilization of stored cholesterol, cholesterol concentrations in adipose tissue remained unchanged during weight loss. Plasma cholesterol levels fell sharply, and microscopic analyses of gallbladder bile revealed cholesterol crystals as well as cholesterol gallstones. Surprisingly, leptin reduced biliary cholesterol secretion rates without affecting secretion rates of bile salts or phospholipids. Instead, cholesterol supersaturation of gallbladder bile was due to marked decreases in bile salt hydrophobicity and not to hypersecretion of biliary cholesterol per se, such as occurs in humans during weight loss. In addition to regulating bile salt composition, leptin treatment decreased bile salt pool size. The smaller, more hydrophilic bile salt pool was associated with substantial decreases in intestinal cholesterol absorption. Within the liver, leptin treatment reduced the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, but it did not change activities of cholesterol 7α-hydroxylase or acyl-CoA:cholesterol acyltransferase. These data suggest that leptin regulates biliary lipid metabolism to promote efficient elimination of excess cholesterol stored in adipose tissue. Cholesterol gallstone formation during weight loss in ob/ob mice appears to represent a pathologic consequence of an adaptive response that prevents absorption of biliary and dietary cholesterol.

Bile is the route for cholesterol elimination from the body, and reverse cholesterol transport is the metabolic pathway for movement of cholesterol from peripheral tissues to the liver for biliary secretion (1). Consistent with their central role in reverse cholesterol transport, high density lipoproteins (HDL) are the principal source of biliary cholesterol (2, 3). In response to biliary secretion of detergent-like bile salt molecules, HDL-derived cholesterol is secreted from hepatocytes into bile together with phospholipid molecules as vesicles (4).

In leptin-deficient obese C57BL/6J (ob/ob) mice, elevated plasma cholesterol levels are due to increased HDL concentrations (5). Silver et al. (6) have demonstrated that defective clearance of HDL particles from plasma by the liver in these animals is reversed by leptin administration. Moreover, hepatocytes cultured from ob/ob mice display alterations in HDL processing and cellular cholesterol distribution, which are also normalized by leptin (7). We have reported abnormalities in biliary lipid secretion in Zucker (fa/fa) rats (8), which become obese because of a missense mutation in the extracellular domain of the leptin receptor that sharply reduces responsiveness to leptin. Although bile salt secretion rates were preserved, biliary secretion rates of cholesterol and phospholipids were severely reduced. Acute (6 h) infusions of leptin at high doses partially restored biliary cholesterol secretion, and the same treatment in lean Zucker (Fa/−−) rats promoted hypersecretion of biliary cholesterol (8). Taken together, these observations suggest that leptin may promote biliary elimination of plasma cholesterol.

In ob/ob mice, the expanded adipose tissue mass represents an important storage depot for cholesterol (9). Because chronic leptin administration to these animals reduces adiposity (10, 11), excess cholesterol must be mobilized for delivery to the liver and secretion into bile. This study was designed to elucidate a regulatory role for leptin in hepatic cholesterol elimination during leptin-induced weight loss in ob/ob mice. Our results reveal that leptin administration leads to a marked increase in the proportion of hydrophilic bile salts in bile, as well as a sharp decline in the size of the circulating bile salt pool. These changes mechanistically account for reduced intestinal cholesterol absorption, which inhibits both assimilation of dietary cholesterol and reabsorption of biliary cholesterol. However, the reduced capacity of hydrophilic bile salts to solubilize cholesterol within the gallbladder results in cholesterol crystallization and gallstone formation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant murine leptin was a gift from Amgen (Thousand Oaks, CA). [4,14C]Cholesterol (50 μCi/mmol), [5,6-3H]β-sitostanol (50 Ci/ mol) from the Jackson Laboratory, Bar Harbor, Maine 04609.

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1 The abbreviations used are: HDL, high density lipoprotein; Acat, acyl-CoA:cholesterol acyltransferase; Cyp7A1, cholesterol 7α-hydroxylase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; VLDL, very low density lipoprotein.
mmol), and oleyl-[1-14C]CoA (55 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). n-NX-Hydroxy-3-[14C]-methylglutaryl-CoA (57 mCi/mmol), n-[5,6-3H]mevalonolactone (58 Ci/mm), and [14C]cholesterol (49 mCi/mmol) were purchased from PerkinElmer Life Sciences. Cholesteryl-[1a, 2a-3H]Holate (24 Ci/mmol) was purchased from Amersham Biosciences. Aluminum and glass slide gel plates were purchased from EM Science (Gibbstown, NJ). The general chemical reagents were obtained from Sigma unless otherwise specified.

**Experimental Design**

**Animals**

Male 8-week-old C57BL/6J mice that were homozygous for the ob mutation were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained in a temperature-controlled room with 12-h day-night cycles (6 a.m. to 6 p.m. light) and were allowed to adapt to the environment for 2 weeks prior to the experiments. The mice were fed a chow diet (LabDiet 5001, PMI Nutrition International Inc, Brentwood, MO) that contained 4.5% fat and <0.02% cholesterol.

**Diet and Leptin Administration**

Starting at 10 weeks of age, ob/ob mice (n = 80) were treated once daily with intraperitoneal injections of leptin dissolved in saline (10 μg/g of body weight) or an equal volume of saline. To achieve isocaloric intake, saline-injected mice were pair-fed to animals that were administered leptin.

**Biliary Lipid Secretion**

The mice were anesthetized with intraperitoneal injections 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 a.m. with a midline abdominal incision. After inspecting the gallbladder for the presence of gallstones, the common bile duct was ligated with silk sutures. Bile flow was diverted for collection by inserting a Teflon Multikanal Plus microplate reader (Eflab, Helsinki, Finland) set to 492 nm. Plasma concentrations of very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL cholesterol were calculated as products of total plasma cholesterol concentrations and relative fast performance liquid chromatography peak areas of respective lipoprotein fractions (8). Hepatic and adipose tissue contents of triglycerides and total as well as free cholesterol were quantified as described previously (8, 17).

**Biliary cholesterol concentrations were determined using the same enzymatic method as for plasma. Biliary bile salt concentrations and compositions were determined by HPLC (8) utilizing glycocholate as an internal standard. The biliary bile salt hydrophobic index was determined according to Heuman (18). Phospholipid concentrations in bile were determined by an inorganic phosphorus procedure (8). The molecular species of phosphatidylcholines in bile were quantified by HPLC (19, 20). The biliary secretion rates of cholesterol, phospholipid, and bile salts (nmol/h) were calculated as products of lipid concentrations and bile flow.

**Enzyme Activities**

Hepatic microsomes were prepared by differential ultracentrifugation (21) and stored at −80 °C. The microsomal protein concentrations were determined according to the Bradford method (22) using a Bio-Rad protein assay reagent and bovine serum albumin as a standard. Hepatic microsomes were used to measure enzyme activities as follows. 3-Hydroxy-3-methylglutaryl (HMG)-CoA Reductase (EC 1.1.1.34)—Activity of HMG-CoA reductase was determined according to Shapiro and Rodwell (23). Briefly, the microsomes (1 mg of protein) were incubated with 7.5 μl (0.33 mCi) of [3H]mevalonate (4.5 μmol of glucose-6-phosphate, 3.6 μmol of EDTA, 0.45 μmol of NADP, 0.3 IU of glucose-6-phosphate dehydrogenase for 15 min at 37 °C. [3H]Mevalonic acid (0.024 GBq) used as an internal recovery standard was added to stop the reaction. Unlabeled mevalonate (1.2 mg/ml) was added to assist with recovery. The samples were further incubated for 30 min at 37 °C to allow for conversion of mevalonic acid to mevalonolactone. After incubation, the microsomal protein was precipitated by centrifugation for 1 min, and an aliquot of the supernatant (100 μl) was applied to aluminum silica gel TLC plates. The plates were developed in acetone:benzene (1:1 v/v) and then subjected to autoradiography. The area containing mevalonate (Rf = 0.6–0.9) was scraped and quantified by liquid scintillation counting using Ecolume (ICN Radiochemicals, Irvine, CA). HMGC-CoA reductase activity was expressed as pmol of [14C]mevalonate produced per min per mg of microsomal protein. Recoveries of [3H]mevalonate ranged from 60 to 90%.

**Cholesterol 7a-Hydroxylase (Cyp7A1)**—Activity of Cyp7A1 was measured according to Jelinek et al. (24). [14C]Cholesterol was used as a substrate and delivered as cholesterol-phosphatidylcholine liposomes (1.8 by weight) that were prepared by sonication. An NADPH-regenerating system (glucose-6-phosphate dehydrogenase, NADP, and glucose-6-phosphate) was included in the assay as a source of NADPH. After addition of glucose-6-phosphate dehydrogenase (0.3 IU), samples containing 1 mg of microsomal protein were incubated for 30 min at 37 °C. The reaction was stopped by addition of 5 ml of chloroform-methanol (2:1 v/v) and 1 ml of 0.05% sulfuric acid. The lower phase was dried under nitrogen, redissolved in chloroform, and then applied to glass silica gel TLC plates together with 7a- and 7b-hydroxysterol standards. TLC plates were developed with ethyl acetate-toluene (3:2 v/v), exposed to iodine vapor to identify standards, and then subjected to autoradiography overnight using XAR-5 film (Kodak). Using the developed film as a guide, the locations of [14C]7a-hydroxycholesterol spots were determined, scraped, and quantified by liquid scintillation counting as described above.

**Acy1-CoA:Cholesterol Acyltransferase (Acat)**—Hepatic Acat activity was measured by incorporation of [14C]oleoyl-CoA into cholesteryl esters in hepatic microsomes according to Smith et al. (25). Microsomes (1 mg of protein) were preincubated at a final volume of 180 μl with albumin (84 mg/ml) in buffer (50 mM KHPO4, 100 mM succrose, 50 mM KCl, 50 mM NaCl, 30 mM EDTA, 2 mM dithiothreitol, pH 7.2) for

**Analytical Techniques**

**Plasma cholesterol and triglyceride concentrations were determined by enzymatic assays using reagents from Sigma and Roche Molecular Biochemicals, respectively. The plasma lipoproteins were fractionated by fast performance liquid chromatography using a Superox 6 HRL/30 column (8). The cholesterol concentrations in fractions (0.3 ml) were determined in individual wells of a 96-well microtiter plate by mixing 150 μl of each fraction plus 200 μl of cholesterol reagent (Sigma). The color was analyzed using a Titerrek Multiskan Plus microplate reader (Eflab, Helsinki, Finland) set to 492 nm. Plasma concentrations of very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL cholesterol were calculated as products of total plasma cholesterol concentrations and relative fast performance liquid chromatography peak areas of respective lipoprotein fractions (8). Hepatic and adipose tissue contents of triglycerides and total as well as free cholesterol were quantified as described previously (8, 17).

**Biliary cholesterol concentrations were determined using the same enzymatic method as for plasma. Biliary bile salt concentrations and compositions were determined by HPLC (8) utilizing glycocholate as an internal standard. The biliary bile salt hydrophobic index was determined according to Heuman (18). Phospholipid concentrations in bile were determined by an inorganic phosphorus procedure (8). The molecular species of phosphatidylcholines in bile were quantified by HPLC (19, 20). The biliary secretion rates of cholesterol, phospholipid, and bile salts (nmol/h) were calculated as products of lipid concentrations and bile flow.
Influence of leptin on body weight in C57BL/6J ob/ob mice. The mice (n = 80) received daily intraperitoneal injections of either saline (○) or leptin (■). The saline-treated mice were pair-fed to mice that received leptin. Vertical arrows indicate the time points at which bile, liver, plasma, and adipose tissue were harvested. The horizontal arrow indicates the 7-day interval during which feces were collected for determination of cholesterol absorption and fecal bile salt excretion. The data symbols represent the means ± S.E.

5 min at 37 °C. This was followed by the addition of 20 µl of oleyl [1-14C]coenzyme A (0.15 GBq/pmol). The reaction was continued for 5 min at 37 °C and then stopped by the addition of 100 µl of chloroform methanol (2:1 v/v). After adding [3H]cholesteryl oleate (0.045 GBq) as an internal standard, the reaction mixture was extracted overnight using 2.5 ml of chloroform-methanol (2:1 v/v) and 1 ml of acidified water. The lower phase was then dried under nitrogen, resuspended in 150 µl of chloroform containing 30 µg of unlabeled cholesteryl oleate, and applied to a glass silica gel TLC plate. The plates were developed in hexane-diethyl ether (9:1 v/v). Cholesteryl oleate was visualized using iodine vapor, scraped from the plate, and quantified by liquid scintillation counting as described above. Recoveries of [1-14C]cholesteryl oleate ranged from 70 to 90%.

Statistical Methods

The data are expressed as means ± S.E. The statistical significance of the difference between means of the experimental groups was tested by Student’s t test. A difference was considered statistically significant for a two-tailed p < 0.05.

RESULTS

Fig. 1 shows trends in body weight during treatment with saline or leptin (10 µg/g). Consistent with well established effects of leptin on body weight in ob/ob mice (10, 11), we observed progressive weight loss over the 28-day treatment period (Fig. 1). Mice that were treated with saline lost weight because of pair feeding. Their dietary intake was restricted by 70–80% during the first 7 days and by 40–60% thereafter. For both groups of mice, weight loss was most rapid during the first 7 days and then slowed to and below baseline, respectively. No differences were observed in either absolute or relative biliary bile salt concentrations. Whereas the biliary

![Graph](https://via.placeholder.com/150)

**FIG. 1.** Influence of leptin on body weight in C57BL/6J ob/ob mice. The mice (n = 80) received daily intraperitoneal injections of either saline (○) or leptin (■). The saline-treated mice were pair-fed to mice that received leptin. Vertical arrows indicate the time points at which bile, liver, plasma, and adipose tissue were harvested. The horizontal arrow indicates the 7-day interval during which feces were collected for determination of cholesterol absorption and fecal bile salt excretion. The data symbols represent the means ± S.E.

**TABLE I.** Biliary lipid compositions and secretion rates

| Day | Concentration | Cholesterol (µmol/L) | Phospholipids (µmol/L) | Bile Salt (µmol/L) | Total lipid concentration (µmol/L) | Secretion rate (µmol/h) | Mean percent |
|-----|---------------|----------------------|------------------------|-------------------|-----------------------------------|------------------------|-------------|
| 0   | Ch            | 0.63 ± 0.03          | 1.47 ± 0.12            | 3.57 ± 0.24       | 23.03 ± 1.09                      | 0.13 ± 0.03            | 25.03 ± 0.03 |
|     | Pl            | 2.84 ± 0.12          | 8.03 ± 0.45            | 19.71 ± 1.11      | 19.71 ± 1.11                      | 0.21 ± 0.04            | 25.03 ± 0.03 |
| 28  | Ch            | 0.21 ± 0.03          | 0.96 ± 0.12            | 4.10 ± 0.06       | 23.03 ± 1.09                      | 0.11 ± 0.03            | 25.03 ± 0.03 |
|     | Pl            | 1.49 ± 0.12          | 3.24 ± 0.12            | 7.26 ± 0.21       | 10.11 ± 0.71                      | 0.21 ± 0.04            | 25.03 ± 0.03 |

The values are the means ± S.E. and were determined from at least five mice/group at each time point.

* p < 0.05, compared with saline treatment.
secretion rates of bile salts and phospholipids decreased to the 
same degree in leptin- and saline-treated mice, the cholesterol 
secretion rates decreased only with leptin treatment.

Microscopic analyses of gallbladder bile from ob/ob mice 
(n = 14) prior to saline or leptin treatment revealed that none 
contained cholesterol crystals or gallstones. However, following 
the period of rapid weight loss at 14 days (Fig. 1), abundant 
cholesterol monohydrate crystals and cholesterol gallstones 
were detected in 8 of 18 (44%) and 2 of 18 (11%) of leptin-
treated mice, respectively. In mice treated with saline (n = 18), 
no cholesterol crystals or gallstones were observed. These find-
ings were unchanged at 28 days.

To gain mechanistic insights into the physicochemical events 
observed by microscopic analysis of gallbladder bile, we ana-
alyzed the molecular species of bile salts in mouse hepatic bile 
(Fig. 2A). As described previously for rat bile, HPLC resolved 
five major bile salt species comprising >90% of biliary bile 
salts, with tauro-α-, tauro-β-, and tauro-ω-muricholate eluting 
in one peak (8). Leptin treatment was associated with substan-
tial increases in the proportions of tauromuricholates and de-
creases in the proportions of taurocholate. In saline-treated 
mice, similar trends were observed, but they were much less 
pronounced. At 14 days, leptin also reduced the contents of the 
hydrophobic bile salts taurochenodeoxycholate and taurode-
oxochole. Fig. 2B displays the hydrophobic index of bile salts. 
The hydrophobic index is a concentration-weighted average of 
HPLC-determined hydrophobicities of individual bile salts 
present in a mixture (18). This parameter allows the overall 
hydrophobicity of a mixture of bile salts to be represented by a 
single value. The bile salt hydrophobic index decreased mark-
edly in leptin-treated mice compared with saline-treated mice.

HPLC resolved nine major peaks corresponding to ten phos-
phatidylcholine molecular species that accounted for >95% of 
phosphatidylcholines (8). Compared with saline treatment at 
28 days, leptin increased the proportion (mol %, means ± S.E.) 
of 16:0–18:2 (saline, 55.5 ± 0.5; leptin, 60.5 ± 0.4) phosphati-
dylcholine molecular species in bile. Decreases were observed 
in the proportions of 16:1–16:1 (saline, 0.76 ± 0.06; leptin, 0.41 ± 0.04), 16:1–20:4 (saline, 5.73 ± 0.31; leptin, 2.86 ± 0.26), 
16:0–20:4 (saline, 9.87 ± 0.18; leptin, 8.61 ± 0.39), 18:0–18:2 
(saline, 5.49 ± 0.12; leptin, 4.56 ± 0.15), and 18:0–18:1 (saline, 
0.50 ± 0.01; leptin, 0.27 ± 0.07) phosphatidylcholines. There 
were no changes in the proportions of 16:1–18:2 (saline, 3.32 ± 
0.04; leptin, 3.06 ± 0.30), 16:0–22:6 (saline, 4.63 ± 0.36; leptin, 
5.40 ± 0.25), and 16:0–18:1 plus 18:0–20:4 (saline, 12.49 ± 
0.37; leptin, 12.42 ± 0.41) phosphatidylcholine molecular 
species.

Fig. 3A shows the effect of leptin on bile salt pool size. 
Compared with more modest decreases that were observed at 
14 days in saline-treated mice, leptin markedly decreased bile 
salt pool sizes at 14 days. At 28 days, there were further 
decreases in the pool sizes of saline-treated mice. However, 
the values did not fall to those observed with leptin administra-
tion. The bile salt species and hydrophobic index of bile salts 
comprising the bile salt pool (data not shown) were similar to those 
of hepatic bile in Fig. 2. The rates of fecal bile salt excretion
were decreased in both leptin- and saline-treated mice compared with base line. The fecal bile salt excretion rate did not differ in leptin-treated mice compared with saline-treated mice. As evidenced by reduced fecal bile salt excretion during the period spanning 14–21 days, the reduction in bile salt pool size because of leptin treatment was largely completed within the first 14 days. Because cholesterol absorption is regulated by both bile salt hydrophobicity (14, 27–29) and pool size (14), we quantified the intestinal cholesterol absorption during the 7-day period beginning at 14 days. Cholesterol absorption was unchanged in saline-treated mice but decreased in mice treated with leptin (Fig. 3C).

Fig. 4 shows the effects of leptin treatment on hepatic lipid contents, as well as on activities of enzymes that control cholesterol metabolism. Hepatic cholesterol concentrations in mg/g liver (Fig. 4A, solid lines) did not change during the 28-day course of the experiment. Because of decreases in liver weights, hepatic cholesterol contents (mg/liver) decreased significantly in both saline- and leptin-treated mice (Fig. 4A, solid lines). Although no differences were observed at 28 days, the hepatic content of cholesterol at 14 days was 50% higher in leptin-treated mice compared with saline-treated mice. Not displayed are the proportions of free cholesterol and cholesteryl esters, which were unchanged. As shown in Fig. 4B, hepatic contents (solid lines) and concentrations (dotted lines) of triglycerides decreased over the course of the experiment. Compared with saline-treated mice, hepatic triglyceride contents and concentrations were higher in leptin-treated mice at 14 days but lower at 28 days.

Fig. 4C presents hepatic enzyme activities at base line and at 14 days, which was the point at which we observed leptin-induced cholesterol crystallization and gallstone formation, as well as major differences in bile salt hydrophobicity (Fig. 2, bottom panel), bile pool size (Fig. 3A), and hepatic cholesterol and triglyceride contents (Fig. 4, A and B). Leptin treatment reduced HMG-CoA reductase activity. However, activities of Cyp7A1 and Acat activity did not differ from base line in either saline- or leptin-treated mice.

To gain insights into plasma sources of biliary cholesterol, we examined the influence of leptin on plasma cholesterol and its distribution among lipoproteins at base line as well as at 14 and 28 days. Fig. 5 illustrates the lipoprotein profiles with fast performance liquid chromatography peak identities assigned in accordance with Silver et al. (6). At 28 days, changes were apparent in both saline- and leptin-treated mice. Whereas the peak area of VLDL cholesterol was unchanged, there were substantial decreases in magnitudes of the LDL/HDL1 and HDL peaks. These changes were more pronounced in leptin-
treated mice. Decreases in LDL/HDL1 fraction in leptin-treated mice (Fig. 5) reflect the disappearance of the HDL1 (6), as confirmed here by the loss of apolipoprotein A-I from this peak by Western blot analysis (data not shown). Consistent with smaller sized particles, the elution volume of the HDL peak was increased by leptin treatment.

Administration of leptin caused significantly greater reductions in total plasma cholesterol concentration (mg/dl, mean ± S.E.) compared with saline treatment after 28 days (base line, 137 ± 3; saline, 85 ± 2; leptin, 63 ± 4). These changes were principally due to decreases in HDL cholesterol concentrations (base line, 113 ± 2; saline, 65 ± 1; leptin, 42 ± 3). Whereas total and HDL cholesterol concentrations were similar at 14 days, there were significant differences in LDL/HDL1 and VLDL cholesterol concentrations at this intermediate time point; LDL/HDL1 cholesterol concentrations decreased in saline-treated mice at 14 days but were unchanged in mice treated with leptin (base line, 22 ± 1; saline, 16 ± 1; leptin, 23 ± 1). VLDL cholesterol concentrations more than doubled in saline-treated mice but increased only modestly in leptin-treated animals (base line, 2.3 ± 0.1; saline, 5.5 ± 0.2; leptin, 3.2 ± 0.1). At 28 days, LDL/HDL1 cholesterol concentrations were decreased to similar extents (saline, 16 ± 1; leptin, 16 ± 1), whereas VLDL cholesterol concentrations were increased to a greater extent by leptin (saline, 3.8 ± 0.1; leptin, 4.8 ± 0.3). With leptin treatment, plasma triglyceride concentrations decreased significantly at 14 days (base line, 52 ± 2; leptin, 26 ± 2) and returned to base line at 28 days. In mice treated with saline, plasma triglyceride concentrations remained unchanged.

Although our experimental design did not accommodate formal calculations of cholesterol fluxes (30), we could estimate the influence of leptin on the elimination of cholesterol from adipose tissues via the liver (26). Cholesterol concentrations in adipose tissue of ob/ob mice (8) were similar in visceral (4.5 ± 0.6 mg cholesterol/g triglyceride) and peripheral fat (4.6 ± 1.9 mg cholesterol/g triglyceride) and were not influenced by saline or leptin treatment. Considering that the lean body mass and water content of ob/ob mice remain constant during weight loss (10, 11), cholesterol mobilized from adipose tissue (Fig. 6, solid lines) was calculated based on weight loss and cholesterol concentration in fat (26). Cholesterol losses from ob/ob mice were estimated to be the sum of cholesterol mobilized from adipose tissue plus from the liver (Fig. 4A). Acknowledging that these assumptions do not account for the possibility that weight loss was accompanied by differential mobilization of cholesterol from tissues other than adipose and liver, Fig. 6 shows that the cholesterol loss was greater from leptin-treated mice than from saline-treated mice during the course of the 28-day treatment period. However, as shown in the inset, the average daily rate of cholesterol loss during the first half of the experiment (i.e. days 0–14) was the same in leptin- and saline-treated mice. During the second half of the treatment period (i.e. days 15–28), the estimated rate of cholesterol loss from leptin-treated animals was 5-fold greater than saline-treated animals (Fig. 6, inset).

**DISCUSSION**

During weight loss, cholesterol that is mobilized when adipose tissue mass contracts must be transported to the liver for elimination via bile (26). Here we have explored molecular mechanisms of hepatic cholesterol elimination using ob/ob mice in which weight loss was induced by chronic leptin administration. Whereas weight reduction was associated with a cascade of metabolic changes, a parsimonious explanation is that bile salt metabolism represents the primary target of leptin action and that other events occur secondarily.

Among the biological effects ascribed to leptin is potentiation of insulin action at the level of the liver (31–33). This activity could explain the marked reductions in both hydrophobicity (Fig. 2B) and size (Fig. 3A) of the bile salt pool in leptin-treated mice. In bile of diabetic rats (34) and mice (28), proportions of more hydrophobic cholate species are elevated compared with hydrophilic muricholates. These changes are reversed by insulin treatment (34). Insulin also down-regulates Cyp7A1 (35, 36), so that suppression of bile salt synthesis may have accounted for the reduction in bile salt pool size. Whereas unchanged Cyp7A1 activities (Fig. 4C) and fecal bile salt excretion rates (Fig. 3B) indicated that bile salt synthetic rates were the same in mice treated with leptin and saline, these measurements were performed at a point in time when the pool size was already beginning to level off in leptin-treated mice (Fig. 3A). This limitation not withstanding, marked reductions in bile salt pool size and hydrophobicity would normally be expected to increase Cyp7A1 activity (37). The absence of Cyp7A1 up-regulation at 14 days may be construed as evidence that leptin acted to suppress bile salt synthesis. Because regulation of bile salt pool is multifactorial (38), additional experiments will be required to ascertain with certainty whether leptin...
FIG. 6. Influence of leptin on cholesterol elimination from ob/ob mice. For mice treated with saline (○) or leptin (■), the solid lines represent estimated cholesterol losses from adipose tissue, which were calculated as products of weight loss and cholesterol contents of adipose tissue. The dashed lines represent the sum of calculated losses of cholesterol from adipose tissue and measured losses from liver (Fig. 4A). The inset displays estimated rates of cholesterol elimination from adipose tissue and liver during the first and second 14-day periods of the experiment. Hatched bars, adipose tissue/saline treatment; black bars, adipose tissue/leptin treatment; open bars, liver/saline treatment; cross-hatched bars, liver/leptin treatment. Because concentrations of cholesterol in liver were higher than in adipose tissue and because liver weights did not decrease at the same rates in leptin and saline-treated mice, cholesterol losses from liver (Fig. 4A) were excluded from calculations of cholesterol eliminated from adipose tissue.

contracts pool size by decreasing bile salt synthesis, by altering expression of protein(s) responsible for enterohepatic cycling (39), or by increasing gallbladder motility (40, 41).

The pronounced reduction in bile salt hydrophobic index in leptin-treated animals largely explains decreases in biliary cholesterol secretion rates (Table I). Secretion rates of cholesterol vary in proportion to the hydrophobicity of the secreted bile salt species (4), and cholesterol secretion rates were highly correlated with bile salt hydrophobicity in ob/ob mice treated with leptin ($R^2 = 0.95$). Consistent with uncoupling of cholesterol secretion from bile salt secretion in Zucker (fa/ fa) rats, bile salt hydrophobic index was poorly correlated with cholesterol secretion rates in saline-treated ob/ob mice ($R^2 = 0.56$). Despite differences in bile salt hydrophobicity, the biliary phospholipid secretion rates decreased in both leptin- and saline-treated animals to similar extents. This is likely attributable to leptin-induced increases in the concentration of 16:0–18:2, the major molecular species of phosphatidylcholine that is secreted into bile. Enrichment of this molecular species within the canalicular membrane would tend to facilitate bile salt-membrane interactions that promote the biliary secretion of phosphatidylcholine-cholesterol vesicles (4, 42).

Enrichment of bile with hydrophilic bile salts alters the phase equilibria of biliary lipids so that crystallization of cholesterol can occur at relatively low molar percentages (12). This explains the mechanism by which bile became more saturated with cholesterol despite lower cholesterol secretion rates. Moreover, phosphatidylcholine species with more unsaturated fatty acyl chains, such as 16:0–18:2, decrease cholesterol solubility in bile (19, 43). Consistent with predictions based on observations in model systems (12), cholesterol nucleation under the current experimental conditions yielded only cholesterol monohydrate crystals and not anhydrous cholesterol crystal habits.

Both bile salt pool size and hydrophobicity regulate intestinal cholesterol absorption in mice. The percentages of cholesterol absorbed vary in direct proportion to bile salt hydrophobicity (14, 27–29) and to bile salt pool size (14). In mice administered leptin, marked decreases in both bile salt pool size and hydrophobicity were accompanied by a pronounced decrease in cholesterol absorption. By providing a likely molecular mechanism, our findings confirm and extend a recent report that cholesterol absorption is inhibited by leptin administration to ob/ob mice (44).

A distinct effect of leptin in ob/ob mice is to increase hepatic HDL clearance (6), as confirmed by this study. Therefore, the similar steady state HDL cholesterol concentrations in leptin- and saline-treated mice following the period of rapid weight loss at 14 days suggest that higher HDL clearance rates in leptin-treated animals were balanced by increased production because of mobilization of cholesterol from adipose tissue. This possibility is supported by the observation that when fat stores were depleted by leptin administration at 28 days (10, 11), HDL cholesterol concentrations decreased to lower levels than in saline-treated animals.

The calculations presented in Fig. 6 suggest that leptin-induced decreases in bile salt hydrophobicity and pool size that occurred largely during days 0–14 (Figs. 2B, and 3A) functioned to promote efficient cholesterol elimination during days 15–28. These changes reduced cholesterol absorption (Fig. 3C) to such an extent that hepatic cholesterol synthesis would have been expected to increase to a rate that exceeds cholesterol elimination from adipose tissue via the liver (Fig. 6, inset) (30). However, adaptive down-regulation of cholesterol synthesis within the liver (Fig. 4C) fully accommodated the flux of additional HDL cholesterol from the periphery, without changing Acac activity (Fig. 4C) or hepatic cholesterol concentrations (Fig. 4A).

In summary, changes in biliary lipid metabolism induced by chronic leptin administration to ob/ob mice reflect an integrated regulatory response that promotes elimination of endogenous cholesterol during weight loss, when the flux of cholesterol from adipose tissue to the liver is increased. A smaller, more hydrophilic bile salt pool functions to inhibit intestinal absorption of dietary cholesterol and reabsorption of biliary cholesterol. The capacity to regulate bile salt hydrophobicity represents an adaptive mechanism that is not observed in human beings, in whom the composition of the bile salt pool does not change during weight loss (26, 45). Consequently, hypersecretion of biliary cholesterol represents the main mechanism by which humans eliminate cholesterol that is mobilized from adipose tissue. When weight loss is sufficiently rapid, cholesterol crystallizes to form gallstones (45–48). By contrast, cholesterol gallstone formation, when it occurs in the setting of
rapid weight loss in leptin-treated ob/ob mice, is a pathologic consequence of the capacity of the mouse to reduce the hydrophobicity of its bile salt pool.

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