Impaired biliary lipid secretion in obese Zucker rats: leptin promotes hepatic cholesterol clearance

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Received 3 February 2001; accepted in final form 13 April 2001

VanPatten, Sonya, Narasimha Ranginani, Sarah Shefer, Lien B. Nguyen, Luciano Rossetti, and David E. Cohen. Impaired biliary lipid secretion in obese Zucker rats: leptin promotes hepatic cholesterol clearance. Am J Physiol Gastrointest Liver Physiol 281: G393–G404, 2001.—Human obesity is associated with elevated plasma leptin levels. Obesity is also an important risk factor for cholesterol gallstones, which form as a result of cholesterol hypersecretion into bile. Because leptin levels are correlated with gallstone prevalence, we explored the effects of acute leptin administration on biliary cholesterol secretion using lean (FA/−) and obese (fa/fa) Zucker rats. Zucker (fa/fa) rats become obese and hyperleptinemic due to homozygosity for a missense mutation in the leptin receptor, which diminishes but does not completely eliminate responsiveness to leptin. Rats were infused intravenously for 12 h with saline or pharmacological doses of recombinant murine leptin (5 μg·kg−1·min−1) sufficient to elevate plasma leptin concentrations to 500 ng/ml compared with basal levels of 3 and 70 ng/ml in lean and obese rats, respectively. Obesity was associated with a marked impairment in biliary cholesterol secretion. In biles of obese compared with lean rats, bile salt hydrophobicity was decreased whereas phosphatidylcholine hydrophobicity was increased. High-dose leptin partially normalized cholesterol secretion in obese rats without altering lipid compositions, implying that both chronic effects of obesity and relative resistance to leptin contributed to impaired biliary cholesterol elimination. In lean rats, acute leptin administration increased biliary cholesterol secretion rates. Without affecting hepatic cholesterol contents, leptin downregulated hepatic activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, upregulated activities of both sterol 27-hydroxylase and cholesterol 7α-hydroxylase, and lowered plasma very low-density lipoprotein cholesterol concentrations. Increased biliary cholesterol secretion in the setting of decreased cholesterol biosynthesis and increased catabolism to bile salts suggests that leptin promotes elimination of plasma cholesterol.

obesity; liver; bile salts; phospholipids; lipoproteins

HUMAN OBESITY IS ASSOCIATED with altered cholesterol homeostasis including increased production and turnover (33, 37), as well as secretion of excess cholesterol from the liver into bile (43). Among the clinical consequences are cholesterol gallstones, which occur with high frequency in individuals who are obese, lose weight rapidly (29), or experience frequent fluctuations in weight (59). Leptin is a 16-kDa circulating hormone that is secreted by adipocytes and plays a critical role in regulation of body weight. Plasma leptin levels are elevated in most obese individuals (10) and are highly correlated with the frequency of gallstone disease in Mexican Americans (14).

Although rarely the cause of human obesity, spontaneous mutations in leptin or its receptor constitute the genetic basis for obesity in several well-established rodent models (17). Zucker rats become obese and hyperleptinemic due to a Q269P missense mutation that is present in all isoforms of the leptin receptor (42). However, leptin receptors harboring the fa mutation appear to retain residual function, as evidenced by blunted physiological responses of obese (fa/fa) Zucker rats to pharmacological doses of leptin (13, 16, 67). Unlike human obesity in which whole body cholesterol production is increased (33, 37), endogenous rates of cholesterol synthesis do not differ in lean and obese Zucker rats (32). Nevertheless, when compared with other monogenic animal models of obesity due to mutations in leptin or the leptin receptor (17), Zucker rats more closely parallel humans, in whom obesity is associated with leptin resistance (10). The aim of this study was to examine systematically the influence of obesity and leptin on biliary lipid secretion using lean (FA/−) and obese (fa/fa) Zucker rats. Our findings demonstrate that obesity in this animal model is associated with a marked impairment in biliary cholesterol elimination. Consistent with a residual capacity of obese Zucker rats to respond to leptin, this defect in biliary lipid secretion was partially reversed by high-dose intravenous leptin. In lean rats, acute leptin administration promoted excess biliary cholesterol secretion. We further show that leptin downregulated cholesterol biosynthesis, upregulated cholesterol catabolism, and decreased plasma very low-density li-
poprotein (VLDL) cholesterol concentrations. Taken together, these findings suggest that an important biological function of leptin is to promote biliary clearance of plasma cholesterol.

MATERIALS AND METHODS

Materials

Recombinant murine leptin was a gift from Amgen (Thousand Oaks, CA). All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. [4-14C]cholesterol (60 mCi/mmol, DuPont) was obtained from New England Nuclear (Boston, MA) and was purified by silicic acid chromatography (49). 3-Hydroxy-3-methyl[3-14C]glutaryl-coenzyme A ([14C]HMG-CoA, 57 mCi/mmol) and [5-3H]mevalonolactone (24 Ci/mmol) were purchased from Amersham (Arlington Heights, IL).

Animals

Lean and obese male Zucker rats (Charles River Laboratories, Wilmington, MA) 11–13 wk of age were maintained on Chow diet with water ad libitum while being subjected to cycles of 12 h light (6 AM to 6 PM) alternating with 12 h dark. After an overnight fast, rats were anesthetized by an intraperitoneal injection of 50 mg/kg body wt of pentobarbital sodium. After an overnight fast, rats were anesthetized by an intraperitoneal injection of 50 mg/kg body wt of pentobarbital sodium. Blood (1 ml) was sampled for baseline measurements in the left jugular vein and advanced into the right atrium (44). Catheters were exteriorized through a peritoneal injection of 50 mg/kg body wt of pentobarbital sodium. Through an abdominal incision, the catheter was placed surgically in the left jugular vein and advanced into the right atrium (44). Catheters were exteriorized through a 1-cm incision on the back of the neck, which was then closed with surgical clips. Blood (1 ml) was sampled for baseline lipoprotein analysis (see Experimental Design), and then catheters were flushed with 1 ml of heparin (100 U/ml) to maintain patency. Rats were allowed to recover for 3 to 4 days before experiments.

Experimental Design

At 6 AM, conscious unrestrained rats were administered either a bolus injection (2 min) of 150 μg of leptin (100 μg/ml in 150 mM NaCl) or the same volume of saline. This was followed by a 6-h period of continuous infusion (Harvard Infusion Pump, Harvard Apparatus, South Natick, MA) of leptin (100 μg/ml in 150 mM NaCl) at 5 μg·kg⁻¹·min⁻¹ or saline at the same flow rate. The infusion was continued while rats were anesthetized with 50 mg/kg body wt of pentobarbital sodium. Through an abdominal incision, the distal common bile duct was ligated. A PE-10 polyethylene cannula (Clay Adams, Becton Dickinson, Sparks, MD) was inserted in the proximal common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula was externalized, and the abdominal incision was closed. Immediately after surgery, 1 ml of blood was removed via the jugular venous catheter, and the infusion of leptin or saline was resumed for the remainder of the experiment. After a 0.5-h recovery period from surgery, bile was collected at 0.5-h intervals for 5 h into preweighed Eppendorf tubes. Rats were fasted during the 12-h time course of these experiments. At the end of the experiment, rats were euthanized by cardiac puncture. Livers were immediately excised, rinsed with 0.15 M NaCl to remove blood, weighed, and then snap-frozen in liquid nitrogen. Livers and bile samples were stored at −80°C before analysis. Samples of blood were anticoagulated with EDTA, and plasma was separated by centrifugation and stored at 4°C for analysis within 24 h. This protocol was approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Analytical Techniques

Biliary lipids. Biliary cholesterol concentrations were quantified by HPLC (66). Phospholipid concentrations in bile were determined by an inorganic phosphorous procedure (4), and bile salt concentrations were measured enzymatically (4). Biliary bile salt species were analyzed by HPLC (45) using a Beckman Ultrahydro ODS column (4.6 mm × 250 mm, 5 μm; mobile phase methanol:0.01 M KH2PO4, 75:25 vol/vol, pH 5.35). Bile salt hydrophobic index was determined according to Heuman (23). Bile flow rates were calculated assuming hepatic bile density of one (9), so that bile volumes were equivalent to weights. Cholesterol, phospholipid, and bile salt secretion rates (μmol·kg⁻¹·h⁻¹) were calculated as products of lipid concentrations and bile flow rates.

Tissue lipids. Hepatic contents of free cholesterol, total cholesterol, and triglyceride were determined enzymatically according to Carr et al. (5). Briefly, frozen samples of liver tissue were extracted in chloroform:methanol (2:1 vol/vol), and phases were separated by addition of 20 vol% of 0.05% H2SO4. Appropriate volumes of the lower organic phase were mixed together in glass test tubes with 1 ml of Triton X-100 dissolved in chloroform (1% wt/vol), dried under a stream of nitrogen, and resuspended in 0.5 ml of H2O. Enzymatic assays were performed by adding 50-μl aliquots of sample to individual wells of a 96-well microtiter plate. Free or total cholesterol was measured by addition of 150 μl of free cholesterol C enzymatic reagent (Wako Chemical, Richmond, VA) or cholesterol high-performance reagent (Boehringer Mannheim/Roche Diagnostics, Indianapolis, IN), respectively. Triglycerides were measured by sequential addition of 75 μl each of the two reagents of the triglycerides-GB reagent kit (Boehringer Mannheim/Roche Diagnostics) as described (5). Plates were incubated at room temperature for 1 h and then analyzed using a TiterTek Multiskan Plus microplate reader (Tina Lab, Finland) set to 492 nm. Hepatic phospholipid concentrations were determined from phosphorus content (4), which was measured after organic extraction.

Plasma lipids. Total plasma cholesterol concentrations were determined by HPLC (66), and triglyceride concentrations were determined enzymatically (Boehringer Mannheim/Roche). Plasma lipoproteins were fractionated by fast-performance liquid chromatography (FPLC) into VLDL, low-density lipoproteins (LDL), and high-density lipoproteins (HDL) using two prepacked Pharmacia Biotech (Piscataway, NJ) Superose 6 HR10/30 columns connected in series (26). Samples (0.2 ml) were applied to columns equilibrated with phosphate-buffered saline (0.15 M NaCl, 2.6 mM KCl, 5 mM Na2EDTA, 3 mM NaN3, and 10 mM phosphate buffer, pH 7.4) and eluted at a flow rate of 18 ml/h. Cholesterol concentrations in fractions (0.3 ml) were determined by mixing 150 μl of each fraction plus 200 μl of cholesterol 50 reagent (Sigma) in individual wells of a 96-well microtiter plate. Color was developed for 10 min at 37°C and then analyzed using a microplate reader set at 492 nm, as described in Tissue lipids. Plasma concentrations of VLDL-, LDL-, and HDL cholesterol were calculated as products of total plasma cholesterol concentrations and relative FPLC peak areas of respective lipoprotein fractions.

Phosphatidylcholine molecular species. Molecular species of phosphatidylcholines in liver and bile were quantified as previously described (8, 41). Briefly, phosphatidylcholines were purified using a Hibar 5-μm silica 4.6 × 250-mm LiChrospher Si-100 HPLC column and a mobile phase consist-
ing of isopropanol-hexane-ethanol-25 mM phosphate buffer (pH 7.0)-glacial acetic acid (495:367:100:57:0.3 by vol). Phosphatidylcholines were hydrolyzed to form diglycerides using phospholipase C from Bacillus cereus (Boehringer Mannheim/Roche). Benzoate derivatives were prepared and then fractionated by reverse phase HPLC (Beckman Ultrasound 5 μm ODS 2.5 x 250-mm column; mobile phase methanol-water-acetonitrile 94:2:9 by vol). Benzoate derivatives were detected by absorbance at 230 nm so that relative peak areas directly represented mole fractions of individual phosphatidylcholine molecular species. Peak identities were established according to Patton and Robins (41). For indeterminant peaks, molecular species were identified by matrix-assisted time-of-flight mass spectrometry (47) using a Voyager Biospectrometry workstation (Perceptive Biosystems, Framingham, MA).

Hepatic enzyme activities. Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation (50), and protein concentrations were determined according to Lowry et al. (30). Cholesterol concentrations in microsomes and mitochondria determined by gas liquid chromatography (72) were incorporated into the calculations of the specific radioactivity of the [4-14C]cholesterol substrate used in determining the catalytic activities of the microsomal cholesterol 7α-hydroxylase and mitochondrial sterol 27-hydroxylase.

**Cholesterol 7α-hydroxylase (EC 1.14.13.17).** The activity of cholesterol 7α-hydroxylase was measured by an isotope incorporation method according to Shefer et al. (50) with some modifications. The reaction mixture (final vol 0.5 ml) consisted of potassium phosphate buffer (100 mM K2HPO4, 0.1 mM EDTA, 5 mM DTT, 30 mM nicotinamide, pH 7.4), [4-14C]cholesterol (5 x 105 dpm), solubilized in 50 μl of 25% (wt/vol) β-cyclodextrin (final concentration 0.8%), and 50–200 μg of microsomal protein. The reaction was initiated by the addition of NADPH or an NADPH-generating system (3.4 mM NADPH, 30 mM glucose-6-phosphate, 0.3 U of glucose-6-phosphate dehydrogenase) and continued for 15 min at 37°C. The reaction was stopped with 0.5 ml of 1 N KOH, 5 μg of butylated hydroxytoluene, and 10 μl of ethanolic [3H] 7α-hydroxycholesterol (1 x 104 dpm/5 μg) was added as an internal recovery standard. After saponification at 37°C for 1 h, sterols were extracted twice with 3 ml of n-hexane, and the extracts were evaporated to dryness under nitrogen. The residue was dissolved in 0.3 ml of n-hexane:2-propanol (97:3 vol/vol) and applied to a silica column (500 mg; Sep-Pak, Waters). After being washed with 1 ml of n-hexane followed by 4 ml of n-hexane:2-propanol (97:3 vol/vol), 7α-hydroxycholesterol was eluted with 3 ml of n-hexane:2-propanol (80:20 vol/vol). It was further isolated by TLC on silica gel plates (Silica Gel 60, EM Science, Gibbstown, NJ) with diethyl ether and quantified by liquid scintillation counting using Ecolume (Silica Gel 60 plates with acetone:benzene (1:1 vol/vol) as the solvent system. The biosynthetic product, [14C]mevalonolactone, was quantified by liquid scintillation counting as previously described.

**Analysis of Biliary Lipid Secretion Rates**

Over the range of bile salt secretion rates in the current study, secretion rates of cholesterol and phospholipids varied linearly as functions of bile salt secretion rates (Fig. 1, A-D). Physiologically, the slopes of these lines, (i.e., ΔCh/ABS or ΔPL/ΔBS) reflect the molecular coupling (a.k.a., linkage; Ref. 21) of cholesterol or phospholipid to bile salts (i.e., moles of cholesterol or phospholipid secreted per moles of bile salt) (9). Cholesterol secretion rates also varied linearly as functions of phospholipid secretion rates (Fig. 1, E-F) so that the molecular coupling of cholesterol to phospholipid is represented by the slope ΔCh/ΔPL (21). Biliary lipid secretion rates for lean or obese rats infused with leptin or saline were pooled before regression analysis (9, 22). Slopes and their standard errors were then obtained by linear regression analyses according to the method of Johansen and Lumry (25).

**Statistical Analysis**

Analysis of variance was employed to detect differences among mean values, whereas analysis of covariance was performed to compare slopes of linear regressions (74). Pairwise differences among means and slopes were assessed according to the Fischer’s protected least squares differences method (15).

**RESULTS**

There were no differences between weights of rats infused with leptin (321 ± 15 g (mean ± SE), lean; 511 ± 64 g, obese) or saline (313 ± 12 g, lean; 512 ± 34 g, obese). As indicated by identical liver-to-body weight ratios for lean (0.034 ± 0.001) and obese rats (0.035 ± 0.001), liver masses scaled in proportion to body weight. Therefore, normalization to either liver or body weight provided an equivalent basis for comparing biliary lipid secretion rates in lean and obese rats. Figure 1 presents biliary lipid secretion rates normalized to body weight for lean and obese rats infused with saline or leptin. Bile salt concentrations and secretion rates were similar in all groups (47 ± 3 mM and 123 ± 6 μmoles·kg−1·h−1, respectively) and decreased threefold to 16 ± 1 mM and 41 ± 2 μmoles·kg−1·h−1, respectively, during the 5-h bile collection period. Results of regression analyses for data in Fig. 1 are presented in Table 1. For lean rats infused with saline
(Fig. 1), there were statistically significant positive correlations between biliary bile salt secretion rates and secretion rates of cholesterol (Fig. 1A) and phospholipid (Fig. 1C), as well as between secretion rates of cholesterol and phospholipid (Fig. 1E). The slope values in Table 1 represent the magnitudes of coupling between secretion of bile salts, cholesterol, and phospholipids in lean rats. In contrast, there was no correlation between bile salt secretion rates and secretion rates of cholesterol (Fig. 1B) and phospholipid (Fig. 1D) for obese rats during saline infusion. This indicated that obesity in Zucker rats is associated with uncoupling of cholesterol and phospholipid secretion from bile salt secretion.

Leptin administration (Fig. 1) in lean rats increased \( \Delta Ch/\Delta BS \) without influencing \( \Delta PL/\Delta BS \) (Fig. 1A and C, Table 1). This was associated with a twofold increase in \( \Delta Ch/\Delta PL \) in lean rats (Fig. 1E, Table 1). Leptin infusion in obese rats resulted in statistically significant correlations between bile salt secretion rates and secretion rates of cholesterol (\( \Delta Ch/\Delta BS \), Fig. 1B) and phospholipid (\( \Delta PL/\Delta BS \), Fig. 1D). There was appreciable data scatter in Fig. 1, B and D, which was reflected in the low \( r^2 \) values of 0.151 and 0.053, respectively (Table 1). To ensure that the apparent influence of leptin was not an artifact of pooling biliary lipid secretion rates in lean (A, C, and E) or obese (B, D, and F) bile fistula Zucker rats. Data points from individual rats are indicated by different symbols. Solid symbols and solid regression lines are lean (\( n = 5 \)) or obese (\( n = 5 \)) infused with saline. Open symbols and dashed regression lines are lean (\( n = 5 \)) or obese (\( n = 8 \)) rats infused with leptin. Results of regression analyses are presented in Table 1. B.W., body wt.

<table>
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<tr>
<th>Phenotype</th>
<th>( \Delta PL/\Delta BS )</th>
<th>( r^2 )</th>
<th>( P )</th>
<th>( \Delta Ch/\Delta BS )</th>
<th>( r^2 )</th>
<th>( P )</th>
<th>( \Delta Ch/\Delta PL )</th>
<th>( r^2 )</th>
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<td>Lean Saline (( n = 5 ))</td>
<td>0.102 ( \pm ) 0.008</td>
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<td>0.0001</td>
<td>0.005 ( \pm ) 0.001</td>
<td>0.38</td>
<td>0.0001</td>
<td>0.047 ( \pm ) 0.008</td>
<td>0.40</td>
<td>0.0001</td>
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<tr>
<td>Lean Leptin (( n = 5 ))</td>
<td>0.095 ( \pm ) 0.010</td>
<td>0.67</td>
<td>0.0001</td>
<td>0.009 ( \pm ) 0.002*</td>
<td>0.39</td>
<td>0.0001</td>
<td>0.095 ( \pm ) 0.013*</td>
<td>0.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>Obese Saline (( n = 5 ))</td>
<td>-0.008 ( \pm ) 0.018</td>
<td>0.005</td>
<td>0.64</td>
<td>-0.001 ( \pm ) 0.001</td>
<td>0.007</td>
<td>0.59</td>
<td>0.054 ( \pm ) 0.003</td>
<td>0.89</td>
<td>0.0001</td>
</tr>
<tr>
<td>Obese Leptin (( n = 8 ))</td>
<td>0.048 ( \pm ) 0.014</td>
<td>0.151</td>
<td>0.0007</td>
<td>0.002 ( \pm ) 0.001</td>
<td>0.053</td>
<td>0.05</td>
<td>0.059 ( \pm ) 0.003</td>
<td>0.82</td>
<td>0.0001</td>
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Values are means \( \pm \) SE. Values determined by linear regression analysis of data in Fig. 1. *Significantly different (\( P < 0.04 \)) from saline infusion as according to analysis of covariance (ANCOVA).
secretion rates, the same effect was demonstrated by determining slope values for each individual rat and then comparing the means of slope values for leptin- vs. saline-infused rats. Due to the data scatter, however, we could not exclude the possibility that only a subset of obese rats were responsive to leptin. As shown in Fig. 1F and Table 1, leptin administration also resulted in a slight (10%), but not significant, increase in the value of ΔCh/ΔPL compared with saline infusion in obese rats.

Five major bile salt species were identified in lean and obese rats (Fig. 2), and these comprised ≥85% of biliary bile salts. Because the HPLC technique utilized in this study did not resolve tauro-α-muricholate and tauro-β-muricholate, these isomers were integrated as a single peak. Figure 2, A–C, compares biliary bile salt species at three time periods during bile collection (i.e., 0.5–1 h, 1.5–2 h and 4.5–5 h). Early during the bile collection period (Fig. 2A), the distribution of biliary bile salt species was similar in lean and obese rats. With increasing time of biliary drainage, the proportion of tauromuricholates increased in obese rats, whereas the proportion of taurocholate decreased (Figs. 2, B and C) so that during the final bile collection period (Fig. 2C), tauromuricholates were significantly higher and taurocholate significantly lower. The hydrophobic index is a calculated concentration-weighted average of hydrophobicities of individual bile salts present in a mixture (23) that allows the overall hydrophobicity of biliary bile salts to be represented by a single value. Figure 2D plots bile salt hydrophobicities as functions of bile salt secretion rates. At early points after bile duct cannulation (i.e., at high bile salt secretion rates), hydrophobic indexes of lean and obese rats were similar. As bile salt secretion rates decreased due to drainage of the endogenous bile salt pool, the mixture of bile salts in bile from obese rats became more hydrophilic as evidenced by a decrease in the hydrophobic index of bile salts. The opposite trend was observed in lean rats in which the hydrophobic index increased at low secretion rates, indicating a more hydrophobic mixture of bile salts in bile. Although leptin infusion did not affect the hydrophobic index of obese rats, it prevented an increase in hydrophobic index for lean rats at low bile salt secretion rates.

Table 2 presents hepatic lipid contents of lean and obese Zucker rats. Contents of cholesterol (total, free,
and esterified) as well as of phospholipid were similar in saline- and leptin-infused lean and obese rats. Hepatic triglyceride contents were sevenfold higher in obese compared with lean rats but were not changed by leptin administration. Analysis by thin-layer chromatography (71) demonstrated no changes in the proportions of phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols, phosphatidylserines, or sphingomyelins (data not shown). Biliary phospholipids were 95% phosphatidylcholines in all groups of rats.

Figure 3 demonstrates the effects of obesity as well as leptin infusion on molecular species of phosphatidylcholines in bile and in liver. HPLC resolved seven major peaks corresponding to eight phosphatidylcholine molecular species, which accounted for >87% of phosphatidylcholines. Unlike bile salts, the molecular species of phosphatidylcholines in biles did not vary during the bile drainage period. The peak representing 16:0–18:1 was not separated from 18:0–22:6 phosphatidylcholine. However, Patton and Robins (41) have shown that the 16:0–18:1 molecular species constitutes ~85 and ~60% of this peak in rat bile and liver, respectively. Compared with biliary phosphatidylcholines in saline-infused lean rats, Fig. 3A demonstrates in saline-infused obese rats a 30% reduction in the major biliary phosphatidylcholine, 16:0–18:2. In these obese rats, there was a reciprocal twofold increase in 16:0–18:1 (18:0–22:6). Figure 3B shows that the same qualitative differences between lean and obese rats were also present in liver. In addition, there was in liver a higher percentage of 18:0–18:1 in obese compared with lean rats. Figure 3C presents the ratios of molecular species in bile normalized to the same molecular species in liver. Data plotted in this format reveal relative enrichment of each molecular species in bile compared with liver. Values that exceed 1 (e.g., 16:0–18:2) are relatively enriched in bile compared with liver, and molecular species with ratios <1 are relatively enriched in liver (e.g., 18:0–20:4). Whereas in lean rats, leptin infusion decreased the proportion of 18:0–18:1 phosphatidylcholine in bile, it did not significantly alter the enrichment of this molecular species in bile. In obese rats, leptin did not significantly change the molar percentages of 16:0–18:2 phosphatidylcholine in liver or bile. However, leptin slightly increased the enrichment of this species in bile compared with liver.

To examine whether cholesterol recruited by leptin for secretion into bile might be derived from newly synthesized cholesterol or cholesterol destined for catabolism into bile salts, we measured hepatic activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and sterol 27-hydroxylase. Figure 4 demonstrates that leptin infusion into lean or obese rats resulted in a 50% reduction in HMG-CoA reductase activity. In contrast, leptin treatment doubled the hepatic activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.

Fig. 3. Molecular species of phosphatidylcholine (PC) in biles and livers of Zucker rats. Rats were infused with saline (lean, hatched bars; obese, crosshatched bars) or with leptin (lean, open bars; obese, solid bars) as described in RESULTS. PC molecular species were quantified by HPLC for biles collected 0.5–1 h after bile duct cannulation (A) and livers harvested upon completion of saline or leptin infusions (B). C: bile-to-liver ratio for each PC molecular species. For values that exceed 1 (dashed line), molecular species were enriched in bile, whereas those that fell below 1 were enriched in liver. PC molecular species are arranged from left to right in order of elution by HPLC (i.e., increasing hydrophobicity). Because 16:0–18:1 and 18:0–22:6 PCs were incompletely resolved, peaks were integrated together and the minor species (18:0–22:6) were listed in parentheses.*P < 0.05, obese (saline-infused) vs. lean (saline-infused) rats; †P < 0.05, leptin-infused vs. saline-infused rats.

Fig. 4. Hepatic activities of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase, and sterol 27-hydroxylase. Rats were infused with saline (lean, hatched bars; obese, crosshatched bars) or with leptin (lean, open bars; obese, solid bars). ‡P < 0.02, leptin-infused vs. saline-infused rats.
in obese rats. In lean rats, the activities of both enzymes also tended to increase in response to leptin, but the differences from saline-infused controls did not reach statistical significance.

Finally, we examined the influence of leptin on plasma cholesterol and its distribution among lipoproteins. For each rat, cholesterol concentrations in plasma and its distribution among lipoproteins were determined at baseline (time 0) as well as 6 and 12 h after the start of a saline or leptin infusion. Figure 5 displays representative elution profiles for plasma lipoproteins from lean and obese rats before infusion with leptin or saline. Peak designations as VLDL, LDL, and HDL are in accordance with Liao et al. (28). Because columns were loaded with identical volumes of plasma (200 μl), quantitative as well as qualitative differences were apparent between lean and obese rats. Consistent with earlier findings (28), each lipoprotein fraction contained more cholesterol in obese compared with lean rats, and this difference was most pronounced for VLDL. In addition, the peak fractions of LDL and HDL for obese rats eluted slightly earlier, consistent with larger particle sizes. Because VLDL eluted at the void volume of the Superose 6 gel filtration columns, potential differences in VLDL sizes could not be resolved.

Figure 6 summarizes results for plasma cholesterol and individual lipoprotein fractions. At each point in time, total plasma cholesterol concentrations (Fig. 6A) were higher in obese compared with lean rats. This was generally the case for VLDL, LDL, and HDL (Fig. 6, B–D), with the exception that all differences did not achieve statistical significance. In lean rats, there was a 60% increase in total plasma cholesterol over the 12-h period of the experiment for both leptin- and saline-infused rats, and this was principally due to an increase in the LDL fraction (Fig. 6C). Leptin infusion had no effect on total plasma cholesterol concentrations in lean or obese rats. However, as shown in Fig. 6B, leptin administration decreased VLDL cholesterol in both lean and obese rats compared with saline infusion. Significant differences were observed in both lean and obese rats at 6 h. This difference was maintained at 12 h for obese but not lean rats. As shown in Fig. 6, C and D, leptin administration did not affect the plasma concentrations of LDL or HDL.

**DISCUSSION**

Even before cloning and characterization of leptin and its receptor, abnormalities in biliary lipid metabolism were reported in genetically obese rats. In the Koletsky corpulent (cp/cp) rat, obesity is due to a null mutation in the leptin receptor (60). Using the SHR/N-corpulent strain of the Koletsky (cp/cp) rat, Turley (61) demonstrated reductions in the capacity of bile salts to promote the biliary secretion of cholesterol and phospholipids. This was attributed in part to depressed hepatic cholesterol synthesis in the SHR/N-corpulent rat strain (62). St. George et al. (56) reported quite different obesity-related changes in the JCR:LA-corpulent rats, which are also derived from the Koletsky rat: biles of obese rats were enriched with cholesterol relative to bile salts and phospholipids. These observations...
notwithstanding, a primary pathophysiological role for leptin in hepatic cholesterol elimination has remained unexplored.

This study was designed to examine the role of leptin in biliary cholesterol elimination using the Zucker rat, in which homozygosity for the fa mutation gives rise to obesity but does not completely eliminate leptin responsiveness. We observed that obesity in Zucker rats was associated with uncoupling of cholesterol and phospholipid secretion from biliary secretion of bile salts. At a dose of leptin that we have shown previously to elevate plasma leptin concentrations to 500 ng/ml compared with basal levels of 3 and 70 ng/ml in lean and obese rats, respectively (44), biliary lipid secretion was partially normalized in obese (fa/fa) rats. This treatment induced hypersecretion of biliary cholesterol in lean (FA/–) rats.

In a previous study on the influence of acute intravenous leptin administration in rats (44), a 6-h infusion period was sufficient to observe changes in hepatic glucose production that were correlated with alterations in gene expression of hepatic glucokinase and phosphoenolpyruvate carboxykinase. To begin to explore the influence of leptin on biliary lipid metabolism, the current study was designed to test for an effect of leptin under similar experimental conditions, in which bile fistulae were placed immediately after a 6-h infusion period. An important limitation of this experimental design is that steady-state conditions with respect to bile acid and cholesterol metabolism (27, 53) may not have been achieved under these conditions. In addition, changes in enzyme activities of cholesterol 7α-hydroxylase, sterol 27-hydroxylase, and HMG-CoA reductase (Fig. 4) may have been indicative of posttranslational regulation. Despite these possibilities, our data are consistent with a key role for leptin in regulating biliary lipid secretion.

Although the hepatocellular mechanisms by which bile salts promote biliary secretion of cholesterol and phospholipid molecules remain incompletely understood, accumulating evidence suggests that physical-chemical events that occur at the canalicular (apical) plasma membrane play a critical role in coupling of biliary lipid secretion. As illustrated in Fig. 7, bile salts are actively secreted into bile by ABCB11 (trivially referred to as the bile salt export pump, Bsep), an ATP-dependent transporter localized to the canalicular membrane (19). Translocation of phosphatidylcholines from inner to outer membrane hemi-leaflet is accomplished by ABCB4 (trivially known as Mdr2), a canalicular ATP-dependent P-glycoprotein (46, 52). Detergent-like bile salt molecules interact with the exoplasmic leaflet of the canalicular membrane and promote formation of vesicles composed of phosphatidylcholines and cholesterol molecules. Current concepts suggest that rates of vesicle formation vary in direct proportion to hydrophobicity of bile salts in bile and in inverse proportion to hydrophobicity of PCs in canalicular membrane. On this basis, decreased bile salt hydrophobicity and increased PC hydrophobicity associated with obesity in Zucker rats may contribute to uncoupling of cholesterol and PC secretion from bile salt secretion. Cholesterol that is incorporated into biliary vesicles and VLDL derives from a common pool within the hepatocyte. As indicated by “X,” we speculate that acute leptin administration decreases VLDL production by decreasing triglyceride synthesis within the liver. Consequently, rate at which cholesterol is incorporated into VLDL is decreased. To maintain constant steady-state cholesterol levels in liver: 1) cholesterol biosynthesis from acetyl-CoA is decreased, 2) cholesterol catabolism to bile salts is enhanced, and 3) cholesterol is rerouted for secretion into bile. In this schematic illustrating metabolic consequences of leptin’s action in liver, relative fluxes of bile salts taken up at sinusoidal membrane and secreted across canalicular membrane are not represented in proportion to thickness of arrows. Contribution of recirculating bile salts to biliary bile salt secretion is ~20-fold greater that of newly synthesized bile salts (3).
promote the formation of biliary vesicles composed of phosphatidylcholines and cholesterol (11).

Bile salt-membrane interactions that promote biliary lipid secretion (Fig. 7) are influenced by the molecular species of bile salts as well as the membrane composition. Accordingly, secretion rates of vesicles into bile vary in proportion to the hydrophobicity of biliary bile salts (9, 12). Variations in bile salt hydrophobicities as functions of bile salt secretion rates (Fig. 2C) might have been expected to produce nonlinear relationships between bile salt secretion rates and secretion rates of cholesterol and phospholipid. Presumably due to data scatter, however, these relationships were best modeled as linear relationships (Fig. 1). Over the range of bile salt concentrations in the current study, a diminished capacity of more hydrophilic bile salts to interact with the canalicular membrane may have contributed to uncoupling of biliary lipid secretion in obese rats (Fig. 1, A and C, Table 1).

Studies using both native (18, 73) and model membranes (7, 34, 65) have suggested that the phospholipid composition of the canalicular membrane may influence bile salt-membrane interactions and biliary lipid secretion. In particular, we have demonstrated that substitution of the more hydrophilic 16:0–18:2 phosphatidylcholine with the more hydrophobic 16:0–18:1 species significantly diminishes interactions between bile salts and model membranes (7). Therefore, depletion of 16:0–18:2 phosphatidylcholine in membranes of obese rat livers and enrichment with 16:0–18:1 phosphatidylcholine (Fig. 3) may also have led to uncoupling of lipid secretion.

Partial restoration of lipid coupling (Fig. 1, B and D, Table 1) in the absence of changes in bile salt hydrophobicity (Fig. 2D) or phosphatidylcholine composition (Fig. 3) suggest that leptin’s acute regulatory effects on biliary lipid secretion are not due to changes in physical-chemical interactions among lipids. Stimulation of ABCB4 activity represents a potential canalicular mechanism by which leptin administration promoted biliary phosphatidylcholine secretion in obese rats. In transgenic mice, the level of ABCB4 expression regulates coupling of biliary phosphatidylcholine secretion to bile salt secretion (55). In these mice, however, cholesterol secretion is not tightly coupled to phospholipid secretion (55). In Zucker rats, there was tight coupling of cholesterol to phospholipid secretion in the absence or presence of leptin infusion (Fig. 1F). Moreover in lean rats, the increase in coupling of cholesterol to bile salt secretion occurred without a concomitant increase in phosphatidylcholine secretion rates. Taken together, these findings argue against regulation of biliary lipid secretion by leptin via ABCB4.

The influence of acute leptin administration on hepatic cholesterol metabolism is depicted in Fig. 7. Free and esterified cholesterol contents of livers from lean or obese rats remained unchanged (Table 2), indicating that leptin did not simply mobilize preformed hepatic stores of cholesterol for secretion into bile. To determine whether leptin might have increased synthesis of cholesterol for biliary secretion, we measured activities of HMG-CoA reductase in liver (Fig. 4). Consistent with an earlier study by McNamara (32), we observed similar specific activities in lean and obese Zucker rats. However, 50% reductions in both lean and obese rats revealed that leptin suppressed rather than increased cholesterol biosynthesis. In the liver, cholesterol is also catabolized by conversion to bile acids. This occurs via a classic pathway for which the microsomal enzyme cholesterol 7a-hydroxylase is rate limiting, and an alternative pathway that is initiated by mitochondrial sterol 27-hydroxylase. Although a leptin-induced decrease in cholesterol catabolism could have led to increased biliary cholesterol secretion, acute leptin administration instead promoted twofold increases in the specific activities of cholesterol 7a-hydroxylase and sterol 27-hydroxylase in obese rats and nonsignificant increases in activities of each enzyme in lean rats (Fig. 4). Therefore, leptin increased cholesterol catabolism in addition to suppressing its biosynthesis.

Decreases in plasma VLDL cholesterol concentrations (Fig. 6B) in response to leptin suggests a probable source of excess biliary cholesterol, as is illustrated in Fig. 7. Elevated levels of VLDL in obese Zucker rats are the result of overproduction of VLDL lipoprotein particles (48, 68) rather than defects in their catabolism (68). Therefore, leptin most likely decreased plasma VLDL cholesterol concentrations by suppressing production. Because VLDL and biliary cholesterol appear to derive from a common hepatic pool (36, 54, 58), the decrease in VLDL production may have, in turn, increased the availability of cholesterol for biliary secretion.

In summary, our findings suggest that leptin plays an integral role in biliary elimination of plasma cholesterol and that uncoupling of cholesterol and phospholipid secretion from bile salt secretion in Zucker (fa/fa) rats represents a combined metabolic defect. Chronic obesity is associated with decreases in bile salt hydrophobicity and increases in phosphatidylcholine hydrophobicity, which favor uncoupling. Although acute administration did not reverse these changes, leptin partially restored coupling in obese animals and promoted hypersecretion of biliary cholesterol in lean rats. Collectively, these leptin-induced changes in lipid metabolism imply an integrated regulatory response to promote cholesterol elimination. Because leptin has been shown to inhibit hepatic triglyceride synthesis (64), we postulate that a primary effect of leptin in livers of Zucker rats may have been to inhibit VLDL formation by limiting triglyceride supply. To prevent accumulation of cholesterol that was no longer packaged and secreted in VLDL, the liver responded by downregulating synthesis, upregulating catabolism to bile acids, and increasing biliary cholesterol secretion. It is important to note that our data do not exclude the possibility that leptin directly regulates hepatic activities of cholesterol 7a-hydroxylase, sterol 27-hydroxylase, and HMG-CoA reductase. Hepatocytes express leptin receptors (6, 75), which mediate insulin-like signaling (75). In primary hepatocyte cultures (1), in the isolated perfused rat liver (35) and in vivo (44),
leptin enhances the inhibitory effects of insulin on glycogenolysis and hepatic glucose production. In hepatocytes, insulin downregulates cholesterol 7α-hydroxylase (63, 69) and sterol 27-hydroxylase (63) while upregulating HMG-CoA reductase (20, 57). Therefore, potentiation of insulin action as observed in our experiments (Fig. 4). Similarly, evidence has been presented that activation of peroxisome proliferator-activated receptor-α downregulates cholesterol 7α-hydroxylase (31, 40) and may alter bile acid composition via sterol 12α-hydroxylase (24). Leptin increases peroxisome proliferator-activated receptor-α expression in adipocytes (70), and its influence in hepatocytes remains unknown. Further investigation will be required to elucidate cellular and molecular mechanisms whereby leptin regulates hepatic sterol metabolism.

The proposed role for leptin in biliary elimination of plasma cholesterol is supported by recent observations in leptin-deficient ob/ob mice, which are hypercholesterolemic principally due to elevations in plasma HDL concentrations (39). Silver et al. (51) have demonstrated that chronic treatment of ob/ob mice with low-dose leptin (i.e., amounts insufficient to reduce body weight) reduced plasma HDL cholesterol concentrations by promoting hepatic clearance. In separate studies, Bouchard et al. (2) showed that the ob mutation confers resistance to cholesterol gallstone formation in otherwise gallstone-susceptible C57BL/6 mice. These observations, taken in context with our current findings and epidemiological data (14), suggest an integral role for leptin in cholesterol homeostasis as well as in the pathogenesis of cholesterol gallstones.

We thank Dr. Michael McCaleb (Amgen, Thousand Oaks, CA) for providing the leptin used in this study.

This study was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-48873 (D. E. Cohen) and DK-26756 (S. Shefer). S. VanPatten was supported by National Institutes of Health Training Grant GM-07491.

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