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Leptin regulates gallbladder genes related to absorption and secretion

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Swartz-Basile DA, Lu D, Basile DP, Graewin SJ, Al-Azzawi H, Kiely JM, Mathur A, Yancey K, Pitt HA. Leptin regulates gallbladder genes related to absorption and secretion. *Am J Physiol Gastrointest Liver Physiol* 293: G84–G90, 2007. First published April 26, 2007; doi:10.1152/ajpgi.00389.2006.—Dysregulation of gallbladder ion and water absorption and/or secretion has been linked to cholesterol crystal and gallstone formation. We have recently demonstrated that obese, leptin-deficient (Lep^{ob}) mice have enlarged gallbladder volumes and decreased gallbladder contractility and that leptin administration to these mice normalizes gallbladder function. However, the effect of leptin on gallbladder absorption/secretion is not known. Therefore, we sought to determine whether leptin would alter the expression of genes involved in water and ion transport across the gallbladder epithelium. Affymetrix oligonucleotide microarrays representing 39,000 transcripts were used to compare gallbladder gene-expression profiles from 12-wk-old control saline-treated Lep^{ob} and from leptin-treated Lep^{ob} female mice. Leptin administration to Lep^{ob} mice decreased gallbladder volume, bile sodium concentration, and pH. Leptin repletion upregulated the expression of aquaporin 1 water channel by 1.3-fold and downregulated aquaporin 4 by 2.3-fold. A number of genes involved in sodium transport were also influenced by leptin replacement. Epithelial sodium channel-α and sodium hydrogen exchangers 1 and 3 were moderately downregulated by 2.0-, 1.6-, and 1.3-fold, respectively. Carbonic anhydrase-IV, which plays a role in the acidification of bile, was upregulated 3.7-fold. In addition, a number of inflammatory cytokines that are known to influence gallbladder epithelial cell absorption and secretion were upregulated. Thus leptin, an adipocyte-derived cytokine involved with satiety and energy balance, influences gallbladder bile volume, sodium, and pH as well as multiple inflammatory cytokine genes and genes related to water, sodium, chloride, and bicarbonate transport.

leptin; obesity; ion transport; cytokines

THE GALLBLADDER HAS BOTH ABSORPTIVE and secretory functions, and alterations in these functions are significant contributing factors in gallstone pathogenesis (1, 3, 13, 18, 19, 26–28, 33). Cholesterol gallstone formation has been associated with the dysregulation of ion-transport systems and water channels, which can subsequently lead to an increase in biliary lipid and calcium concentrations (1, 3, 29). In addition, gallbladder ion transport changes sequentially in gallstone formation (3, 16, 19, 28). In the early precrystal stage, sodium absorption (mucosa-to-serosa) is increased, but it is decreased during the later crystal stage. During this later stage, both Na^+ and Cl^- serosa-to-mucosa fluxes are increased. These changes in ion transport are likely to be mediated, in part, through the sodium/hydrogen exchanger (NHE) and the bicarbonate exchangers (H^+ or Cl^-/HCO_3^-) as well as Cl^- channels (13). Increased gallbladder absorption of water during the early stages of gallstone formation may increase solute concentration and promote cholesterol crystal nucleation (34), whereas enhanced secretion of calcium and mucin can also promote nucleation.

Obesity is also a known contributing factor to cholesterol gallstone formation, with obese individuals having a relative risk of 3.7 of developing gallstone disease (17). The etiology of cholesterol gallstone disease is multifactorial, involving a complex interaction between both environmental and genetic factors including age, race, gender, parity, diet, diabetes, and family history as well as obesity (16). The formation of cholesterol gallstones requires four pathophysiological conditions, including increased biliary secretion of cholesterol (producing cholesterol-supersaturated bile), nucleation and growth of cholesterol monohydrate crystals, decreased biliary motility, and altered gallbladder absorption/secretion.

Leptin, the protein product of the *ob* gene, is a small peptide hormone produced primarily by adipose tissue and is highly correlated with total body mass (35). Recessive mutations in the mouse obese (*ob*) and diabetes (*db*) genes result in weight gain and diabetes similar to human obesity. Leptin-deficient (Lep^{ob}) and leptin-receptor deficient (Lep^{db}) mice have similar phenotypes, weighing 2.5–3.0 times more than normal mice and having 5 times the body-fat content. In addition, Lep^{ob} mice exhibit hyperphagia, hyperglycemia, hormonal imbalances, and decreased immune function. These mice are also hypometabolic and hypothermic.

We have recently reported that both leptin-deficient and leptin-resistant mice, as well as nonobese diabetic mice, have increased gallbladder bile volume, possibly due to alterations in gallbladder absorption and/or secretion (6, 7, 8, 10, 31, 32). In addition, leptin-deficient obese mice have decreased gallbladder contractility, an indication of gallbladder stasis, and shortened cholesterol crystal observation time, suggesting the presence of increased cholesterol crystal pronucleators (4, 5). We have also shown that leptin-deficient mice have decreased gallbladder smooth muscle response to excitatory stimuli such as acetylcholine and cholecystokinin (6, 8) and that leptin replacement ameliorates this response (23). Therefore, we hypothesized that administration of leptin to leptin-deficient mice would alter gallbladder absorption and secretion by mediating water and ion flux across the gallbladder epithelium.

MATERIALS AND METHODS

Animals and Surgical Procedures

Female C57Bl/6J-lep^{ob} obese mice (n = 56) aged 7 wk were obtained from The Jackson Laboratory (Bar Harbor, ME). On arrival,

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mice were placed on a standard-chow diet and were allowed to acclimate for 1 wk before the start of the experiment. Mice were housed four to a cage, with free access to water in a temperature- (22°C) and light-controlled (12:12-h light/dark cycle) environment. For leptin replacement, mice received daily intraperitoneal injections of either recombinant murine leptin (R&D Systems, Minneapolis, MN) at a dose of 5 μg/g body wt or saline as a control for 4 wk. All animals were weighed daily. At 12 wk of age, mice were fasted overnight with free access to water. The following morning, the mice were anesthetized with ketamine-xylazine (50 mg/kg ip ketamine, 15 mg/kg ip xylazine) and were weighed, and they underwent cholecystectomy with aspiration of gallbladder bile. Gallbladders were then immediately frozen in liquid nitrogen and stored at −70°C for subsequent analysis. Whole blood was collected from the right ventricle. To obtain sufficient total RNA to perform the desired analyses, three different cohorts of mice were necessary.

Study cohort I. This group consisted of mice ($n = 24$, $n = 12$ each of saline- and leptin-treated animals) that were utilized for the isolation of the total RNA that was applied to microarrays and for serum and bile analyses.

Study cohort II. This group consisted of mice ($n = 20$, $n = 10$ each of saline- and leptin treated animals) that were treated identically to *cohort I* but were utilized solely for verification of gene-expression profiles.

Study cohort III. This group consisted of mice ($n = 12$, $n = 6$ each of saline- and leptin-treated animals) that were weight-matched and pair-fed to control for food intake. These mice were treated as in *cohorts I* and *II* above, with the exception that they were individually housed and were also utilized solely for verification of gene-expression profiles. Indiana University School of Medicine and the Medical College of Wisconsin Animal Care and Use Committees approved all protocols.

Serum and Gallbladder Bile Analyses

Whole blood was centrifuged at 15,000 rpm for 5 min to obtain serum, which was stored at −20°C until further analyses. Aspirated gallbladder bile volume was measured. Serum and gallbladder bile sodium and potassium levels were determined by flame photometry, and bile pH was measured by standard methods.

Microarray Expression Profiles

Arrays, sample preparation, and hybridization. Three gallbladders of mice from *cohort I* were pooled for total RNA isolation by using an RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. Affymetrix oligonucleotide microarrays (mouse genome, 430 2.0 arrays; Affymetrix, Santa Clara, CA), representing 39,000 transcripts, were used to compare gallbladder gene-expression profiles from saline-treated and leptin-replaced mice. The procedures for Affymetrix gene chips beginning with first-strand cDNA synthesis were conducted by Genome Explorations (Memphis, TN). Four RNA pools for each treatment were used, for a total of eight hybridizations. As a control, the same amount of RNA was used on each chip.

Data and statistical analyses. The resulting data were analyzed by using Gene Chip Operating Software (GCOS). The GCOS statistical algorithm calculates signal intensity, probe set detection, gene-expression change, and signal log ratio. The mean of the four saline-treated groups was compared with the mean of the four leptin-replaced groups. The data were adjusted so that the average intensity was the same as the baseline array. The data were also normalized by using global scaling, where the data are multiplied by a scaling factor to make the average intensity equal to a defined target intensity. The average target intensity was set to 250 fluorescent units. The statistical significance of expression changes was determined by using Welch's *t*-test. Averages were expressed as means ± SE. The raw data from GCOS is publicly available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/ provided by the National Institutes of Health, Bethesda, MD), and the group series number is GSE3293.

Real-Time PCR

Total RNA was isolated from pooled gallbladders of mice from *cohort II* and individual gallbladders from mice in *cohort III* by using an RNeasy Mini kit according to the manufacturer's recommendations. RNA was reverse transcribed by using Super Script III Platinum Two-Step qRT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). FAM-labeled primers (LUX primers; Invitrogen, Carlsbad, CA) specific for each of the genes of interest are shown in Table 1. Real-time PCR reactions were carried out on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using Platinum Quantitative PCR SuperMix-UDG according to the manufacturer's recommendation. Simultaneous real-time reactions were carried out for 18S RNA and genes of interest in parallel wells. Relative fold change is expressed as the mean difference between saline- and leptin-treated gallbladder RNA. Due to the amount of RNA obtained, real-time PCR was carried out on only a select number of genes.

Statistical Analyses

Results are expressed as means ± SE. Statistical differences for body weight, gallbladder volume, serum, and bile analyses were determined by Student's *t*-test. $P < 0.05$ was employed as the nominal criterion of statistical significance.

Table 1. *Oligonucleotide pimer sequences used in real-time PCR studies*

Gene Name	Accession Number	Oligo	Strand	3′ Location	Product Size
Aquaporin 4	BB193413	GAT CTG GCT TTC AAA GGC GtC	Forward	33	65
		TGC AGC TCC GTC ACT CAT GC	Reverse	53	
Carbonic anhydrase 4	NM_007607	GGT TCA CCT TTG TCC TAG CAG tG	Reverse	212	75
		GGC CTG GAG CCT GTA AGG AG	Forward	185	
Epithelial sodium channel-α	AF 112185	AGG TCT TGC TCC TTG AAA TTG tTG	Reverse	115	65
		ACG TCT CCA ACT CAC CGA AGG	Forward	101	
Interferon-induced protein	NM_008331	CTC AGA AGC TCA GGC TTA CGt TG	Forward	421	85
		TGG GCA TTC CAT GCT GTA AG	Reverse	458	
Leptin receptor	NM_146146	AGG AGC AAG AGA CTG GAT GGt TC	Forward	941	70
		TGG TAA AGA CTT GAG GTG AAC TCC	Reverse	959	
TNF-β-induced protein 2	NM_009396	TGA GCA ACG AGG AGC TGG tG	Forward	444	69
		CGC AAA GCA GCA CGT ACA GA	Reverse	468	
Cytokine B subfamily 5	NM_009141	AGC ATA TCC CGA GCT GGt G	Forward	39	65
		AGC TGG AGG CTC ATT GTG GA	Reverse	60	
NHE3	BB805362	GAA GGC ATT TCC TTA CAG TGG tG	Forward	297	129
		GCC AAT CGG CCT CAT CCT T	Reverse	214	

t, Location of the FAM-labeled nucleotide.

RESULTS

Leptin Alters Body Weight, Gallbladder Volume, Serum Leptin, Bile Sodium, and pH Levels

Body weights of mice in both the saline-treated and leptin-replaced groups were similar at the start of the experiment. As expected, following leptin replacement, body weights decreased 31% over the course of the experiment compared with saline-treated controls (33.8 ± 0.9 vs. 48.9 ± 0.9 g; $P < 0.001$). Data for body weight, gallbladder volume, serum leptin, bile sodium, and bile pH for *cohort I* are shown in Table 2. At the time of death, gallbladder volumes of leptin-replaced mice also were significantly lower than saline-treated controls (17.2 ± 2.6 vs. 42.2 ± 3.9 μl; $P < 0.001$). Serum leptin levels were significantly greater in the leptin-replaced mice compared with the saline-treated controls (70.32 ± 8.6 vs. 0.63 ± 0.04 ng/ml). Similar results were obtained for body weight and gallbladder volume for mice in *cohort II* (data not shown). Body weights in the pair-fed control group (*cohort III*) decreased 15% over the course of the experiment compared with saline-treated controls (26.9 ± 1.0 vs. 31.8 ± 0.9 g; $P < 0.01$). Gallbladder volumes were also significantly lower than saline-treated controls (14.7 ± 0.9 vs. 32.2 ± 6.3 μl; $P < 0.03$).

Gallbladder bile sodium concentrations were significantly (217 ± 8 vs. 246 ± 2; $P < 0.01$) lower in the leptin-replaced Lep^{ob} mice than in the saline-treated controls. Bile potassium concentrations were not significantly different (data not shown). Serum sodium and potassium levels showed no difference with leptin replacement (data not shown). Gallbladder bile pH was significantly decreased by one pH unit in mice treated with leptin compared with the saline-treated controls (8.0 vs. 7.0; $P < 0.0001$). A summary of these changes is depicted in Fig. 1.

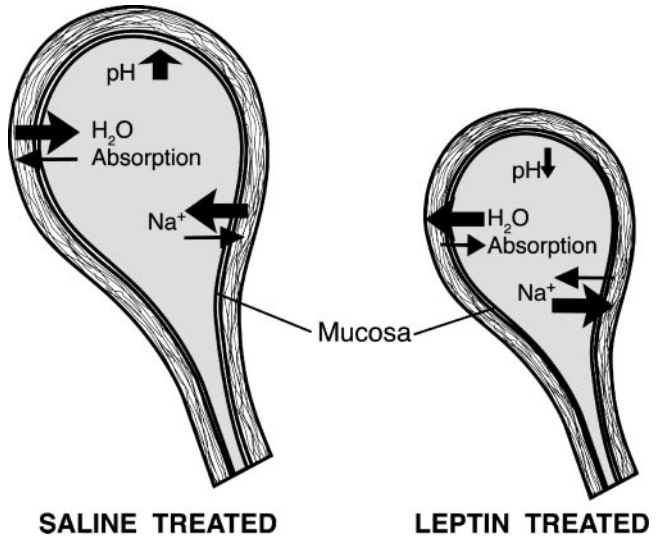

Fig. 1. Leptin administration decreased gallbladder volume, bile sodium concentration, and pH.

Leptin Mediates Expression of Genes Involved in Absorption and Secretion

The set of genes with altered gallbladder expression due to leptin replacement represented a broad spectrum of functional classes, including intermediary metabolism (particularly fatty acid metabolism), signal transduction, transcription factors, and intracellular transport. Analysis of the genes related to lipid metabolism, gallbladder motility, and cholesterol crystal formation is the subject of another report (9). Of the 39,000 genes represented on the arrays, our screening yielded 3,432 differentially regulated genes. Microarray hybridization indicated that 1,795 of these genes were upregulated by leptin, whereas the rest were downregulated (data not shown).

Using microarray analysis, we have identified a number of differentially expressed genes involved in absorption and secretion functions of the gallbladder (Table 3). Leptin was found to downregulate the expression of the water channel aquaporin (AQP) 4 by 2.3-fold in the gallbladder of leptin-replaced mice compared with saline-treated controls. This observation was confirmed by real-time PCR, which indicated a 4-fold decrease and a 1.7-fold decrease in the pair-fed controls. However, the AQP1 water channel was modestly upregulated by 1.3-fold in the gallbladders of leptin-replaced mice compared with saline-treated controls. In addition to water channels, a number of ion transporters were also regulated in the gallbladder following leptin replacement. Several of these were moderately downregulated. Epithelial sodium channel-α was downregulated twofold in gallbladders of leptin-replaced mice. This finding was confirmed by real-time PCR, which also indicated a 2-fold decrease and an 11.1-fold decrease in the pair-fed controls. NHE1 and NHE3 were also downregulated by 1.6- and 1.3-fold, respectively, in the leptin-replaced mice. For NHE3, this observation was confirmed by real-time PCR and in the pair-fed control group. In addition, other ion transporters, including Na^+-K^+-ATPase transporter a1 polypeptide and chloride channel 5, were downregulated by 1.6- and 1.5-fold, respectively, in leptin-replaced mice compared with saline-treated controls. On the other hand, the Na^+-K^+-$2Cl^-$ cotransporter solute carrier 12a2 was found to be upregulated by 1.9-fold in the gallbladders of leptin-replaced mice compared with saline-treated controls.

Genes involved in bicarbonate metabolism and transport were upregulated in the gallbladder following leptin replacement. Carbonic anhydrase (CA)-IV, which catalyses the hydration of carbon dioxide and the dehydration of bicarbonate, was upregulated 3.7-fold following leptin replacement. This observation was confirmed by real-time PCR, which indicated a 14.8-fold increase and a 2.5-fold increase in the pair-fed control group. In addition, the Na^+-HCO_3^- transporter solute carrier 4a2 was found to increase 1.4-fold in the gallbladders of leptin-replaced mice compared with saline-treated controls.

Table 2. *Gallbladder bile analyses*

Treatment	Weight, g	GBV, ml	Na^+, meq/l	pH
n	12	10–11	7	8
Saline	48.9±0.9	42.2±3.9	246±2	8.0±0.13
Leptin	33.8±0.9	17.2±2.6	217±8	7.0±0.14
P value	<0.001	<0.001	<0.01	<0.0001

Values are means ± SE; *P* value was determined by Student's *t*-test. GBV, gallbladder volume.

Table 3. *Differentially expressed genes related to absorption/secretion in the gallbladder following leptin administration*

Gene	Accession Number	Microarray Fold Change	Real-Time Fold Change	Pair-Fed Control Fold Change	*P* Value	Related Pathway/Function
Genes exhibiting upregulation by leptin						
Aquaporin 1	NM_007472.1	1.3	NA	NA	0.020	Water transport
Carbonic anhydrase IV	NM_007607	3.7	14.8	2.5	0.008	Bicarbonate metabolism
Cytokine B subfamily 5	NM_009141	109.4	27.9	8.4	0.010	Inflammatory response
Cytokine-inducible SH-2 protein	NM_009895.1	1.7	NA	NA	0.007	Regulation of signal transduction
IL-1β	BC011437	2.7	NA	NA	0.001	Inflammatory response
IL-1 receptor antagonist	M57525	3.2	NA	NA	0.001	Inflammatory response
IL-2 receptor	L20048	4.6	NA	NA	0.001	Inflammatory response/signal transduction
IL-7 receptor	NM_008372	2.3	NA	NA	0.003	Inflammatory response/signal transduction
INF-induced protein	NM_008331	5.3	4	NA	0.020	Immune response
INF receptor 2	BB522265	1.9	NA	NA	0.001	Signal transduction
INF-α inducible GTPase 1	NM_021792.1	3.3	NA	NA	0.002	Cytokine-mediated signaling pathway
INF regulatory factor 2	NM_008391.1	2.1	NA	NA	0.001	Immune response
Leptin receptor	NM_146146	1.8	1.1	1.3	0.008	Regulation of metabolism/signal transduction
Sodium bicarbonate transporter Slc 4a2	NM_009207.1	1.4	NA	NA	0.040	Bicarbonate transporter activity
Sodium-potassium-choloride cotransporter	BG069726	1.9	NA	NA	0.00002	Ion transport
TNF-α-induced protein 2	NM_009396	3.9	5.6	1.7	0.00005	Angiogenesis
TNF ligand superfamily 13	NM_023517	4.3	NA	NA	0.002	Immune response
TNF 1 receptor	M59378.1	1.5	NA	NA	0.002	Cytokine-mediated signaling pathway
Genes exhibiting downregulation by leptin						
Aquaporin 4	BB193413	2.3	4	1.7	0.008	Water transport
Na^+-K^+-ATPase a1 polypeptide	BC025618	1.6	NA	NA	0.005	Cation transport
Chloride channel 5	BB794830	1.5	NA	NA	0.008	Chloride transport
Epithelial sodium channel α	AF112185	2.0	2	11.1	0.002	Sodium ion transport
NHE1	Al323572	1.6	NA	NA	0.030	Sodium ion transport
NHE3	BB805362	1.3	1.5	1.7	0.040	Cell ion homeostasis

All microarray fold changes were determined by microarray hybridization ($n = 4$) pools from 3 mouse gallbladders/pool. *P* value indicating significant changes in leptin-treated mice compared with saline-treated controls was determined by Welch's *t*-test; NA, not analyzed; IL, interleukin; INF, interferon; TNF, tumor necrosis factor; NHE, sodium/hydrogen exchanger.

Leptin Upregulates Cytokine Genes

The expressions of a number of genes involved in inflammatory responses were upregulated in the gallbladders of Lep^{ob} mice following leptin replacement (Table 3). These genes include members of the cytokine, interleukin (IL), interferon (INF), and tumor necrosis factor (TNF) families. Of particular interest is cytokine B, which was upregulated 109.4-fold in the gallbladder following leptin replacement. A 27.9-fold increase was obtained by real-time PCR, and an 8.4-fold increase was obtained in the pair-fed control group, thus corroborating a substantial fold increase of this cytokine. Cytokine-inducible SH-2 containing protein was only modestly upregulated by 1.7-fold. However, IL-1β, IL-1 receptor antagonist, IL-2 receptor, and IL-7 receptor were moderately upregulated in the gallbladder following leptin replacement. Members of the INF family also were upregulated following leptin replacement. These genes include INF receptor 2, INF-induced protein, INF-α inducible GTPase 1, and INF regulatory factor 2. Leptin replacement also led to significant fold increases in the gallbladder of TNF-α-induced protein 2, TNF ligand superfamily 13, and TNF 1 receptor. The 3.9-fold increase microarray change in TNF-α induced protein 2 was confirmed by a 5.6-fold real-time PCR increase and a 1.7-fold increase in the pair-fed control group. Leptin replacement also increased the leptin receptor by 1.8-fold by microarray and 1.1- and 1.3-fold increases determined by real-time PCR for *cohorts II* and *III*, respectively.

DISCUSSION

In our analysis of the microarray data, we used a hypothesis-driven approach that focused on the expression of genes in functional areas known to influence obesity- and/or diabetes-linked gallstone pathogenesis. These areas included gallbladder motility, nucleation, cholesterol supersaturation of bile, and absorption/secretion. This approach allowed us to define the relationship of leptin and those genes involved in gallstone formation. This study focuses on the genes involved in gallbladder absorption and secretion. Alterations in their expression or regulation by leptin may alter gallbladder ion and water absorption/secretion and thereby influence gallstone formation. In a separate study (9), we have documented the influence of leptin on genes related to gallbladder motility, cholesterol crystal pro- and antinucleators, and gallbladder lipid metabolism.

Masyuk et al. (12) described water transport by gallbladder epithelia as having three main characteristics: *1*) water is

absorbed isosmotically, such that the osmolality of the solution absorbed is the same as that of the luminal solution; *2*) water absorption can occur against a chemical gradient from a concentrated to a dilute solution; and *3*) the net transport of water is coupled to NaCl transport in the same direction. Currently, it is believed that Na^+ enters the apical membrane of gallbladder epithelial cells by way of a NHE, whereas a Cl^-/HCO_3^- exchanger transfers a Cl^- into the cell in exchange for one HCO_3^-. Water then follows passively across the cell.

Water-selective channels AQP1 and AQP8 have been localized in human and mouse gallbladder epithelium (2, 14, 21, 33, 34), suggesting a role in gallbladder secretion and bile concentration. AQP1 and AQP8 were both detected in the apical membrane; however, AQP1 was also present in the basolateral membrane and in vesicles located in the subapical cytoplasm. How aquaporins are involved in the regulation of water absorption/secretion in the gallbladder is currently unknown. However, one hypothesis is that osmotic water would cross the apical membrane through AQP8 and AQP1. The presence of two distinct aquaporins implies that they may be involved in both absorption and secretion of water. van Erpecum et al. (34) recently showed that the expression of AQP1 and AQP8 decreased coincidentally with the concentrating ability of the gallbladders of C57L mice on a lithogenic diet. These data suggest a potential relevance for gallstone formation.

Of particular interest in the current study, leptin replacement, which significantly decreased gallbladder volume in the Lep^{ob} mice, moderately enhanced the expression of AQP1 by 1.3-fold. AQP8 was not detected; thus its expression is likely not influenced by leptin. In addition, leptin replacement downregulated AQP4 by 2.3-fold, which to our knowledge has not been previously reported to be expressed in the gallbladder. AQP4 may function primarily as a secretory water channel. These data suggest that leptin may alter gallbladder volume by mediating gallbladder absorption/secretion of water with a net increase in water absorption through aquaporin channels.

During the fasting state, gallbladder sodium absorption is primarily mediated through the apically restricted NHE (13). Na^+ is absorbed, and bile is acidified (24, 29). NHE is a membrane protein that is regulated by protein kinase C-α and facilitates the electroneutral exchange of extracellular Na^+ for intracellular H^+ (20). The associated Cl^- transport is likely to occur through Cl^-/HCO_3^- exchangers and Cl^- channels. Postprandial gastrointestinal hormones such as vasoactive intestinal peptide and serotonin can increase cAMP levels, which inhibit apical NHE and Cl^-/HCO_3^- exchangers, resulting in the inhibition of net NaCl entry (20). Narins et al. (18) demonstrated that NHE2 and NHE3 are involved in vectorial Na^+ transport in the gallbladder in the prairie dog. In subsequent studies, they showed that gallbladder Na^+ absorption is increased before crystal formation due, in part, to an increased NHE activity and that a shift in NHE activity occurs from NHE2 to NHE3 as the prairie dogs progressed through the stages of gallstone formation from no stones to crystals and finally gallstones (19).

In the current study, NHE1 and NHE3 were moderately downregulated with leptin replacement, whereas NHE2 was not detected. In addition, epithelial sodium channel-α and chloride channel 5 were downregulated by 2.0- and 1.5-fold, respectively, following leptin replacement. Moreover, Na^+ levels in the gallbladder bile also were significantly decreased with leptin replacement compared with the saline-treated group. These data suggest that leptin may influence gallstone formation by altering Na^+ levels in the gallbladder bile.

Apical Na^+/H^+ exchangers also are believed to be involved in lowering gallbladder bile pH (22). Acidification of gallbladder bile is an important factor in preventing calcium carbonate precipitation and gallstone formation. CAs, which catalyze the hydration of carbon dioxide and the dehydration of bicarbonate ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$), also may play a role in the acidification of bile (30). The apical membrane-associated CA-IV, along with CA-II, is believed to function together with the NHE in the gallbladder epithelium, resulting in net acidification of the bile. In the experiment presented herein, leptin replacement significantly increased the expression of CA-IV by 3.7-fold. CA-II was not affected by leptin. These data suggest that leptin may influence gallstone formation by lowering biliary pH as well as Na^+ levels and may thus prevent calcium precipitation.

A number of inflammatory cytokines, including genes of the IL-1 and TNF-α families, were upregulated following leptin replacement. Leptin's functions are not restricted to energy homeostasis; increasing evidence suggests that leptin also functions as a proinflammatory cytokine. Leptin has been demonstrated to promote Th1 lymphocyte activities (11). Serum leptin levels also are acutely increased by many acute-phase factors, such as TNF, IL-1, and IL-6 (11). These factors can, in turn, stimulate adipocytes to secrete leptin, which can further promote the inflammatory response. Inflammation of the gallbladder wall is an early characteristic of animal models of gallstones (25). Inflammation has been detected as soon as luminal crystals appear. Rege and co-workers (25, 26) also have shown that this inflammation is associated with increased myeloperoxidase and IL-1 activity in the gallbladder wall. In addition, they have demonstrated that IL-1α and TNF-α decrease mucosal-to-serosal and net Na^+ and Cl^- fluxes and increase serosal-to-mucosal movement of sodium across the gallbladder epithelium. These observations suggest that alterations in gallbladder absorptive/secretive function, and thus gallbladder volume, due to inflammation in the gallbladder wall may contribute to gallstone formation.

Although in the current study leptin replacement upregulated TNF-α and IL-1α, gallbladder volume was decreased or normalized. Leptin is a pleiotropic hormone whose multiple effects could influence any one or all of the numerous factors that affect gallbladder volume in addition to absorption and secretion. These factors include the hepatic production of bile, hormonally and neurally mediated resting tone of the gallbladder, and sphincter of Oddi function.

Other cytokines that were upregulated by leptin replacement include cytokine B subfamily 5 (Scyb5). Scyb5 is a member of the CXC chemokine family and is believed to stimulate the infiltration of lymphocytes into tissue from peripheral blood and to function as a mediator of inflammation (15). A number of members of the IFN family also were upregulated with leptin replacement. Little is known regarding the roles of Scyb5 or IFNs in the gallbladder. However, they likely contribute to the proinflammatory state of the gallbladder that is associated with gallstone formation.

Unlike the leptin-deficient murine Lep^{ob} model utilized in these studies, human obesity is usually a hyperleptinemic state that also is considered to be a chronic proinflammatory state. Thus leptin levels are associated with several proinflammatory

cytokines (11). Although administration of leptin to the leptin-deficient Lep^{ob} mice physiologically decreased body weight and reduced gallbladder volume, leptin administration may have created a hyperleptinemic state that induced proinflammatory cytokines. Recent human interventional studies involving administration of rmetHuleptin to normal and obese humans demonstrated that increasing leptin levels to high physiological or pharmacological levels does not alter proinflammatory cytokine levels in subjects with leptin sufficiency or excess (obese) (11). Thus the main role of leptin may be to regulate immune function in leptin-deficient and not leptin-sufficient states in humans.

Leptin replacement led to a significant 31% decrease in body weight compared with the saline-treated group. A pair-fed control group (*cohort III*) was included. Although pair fed for 1 mo, the leptin-replacement group's average body weight remained ~15% lower than that of the saline control, and gene-expression profiles of all the genes measured in *cohort III* verified the results obtained in *cohort I*, suggesting that leptin influences body weight and gene expression regardless of caloric intake.

Limitations of our study include the fact that the data obtained are based solely on mRNA levels as determined by microarray and quantitative PCR. Many genes undergo post-transcriptional regulation, and thus our data only indicate a subset of gallbladder genes whose regulation is influenced by leptin. In addition, the leptin-replacement model utilized in these studies does not reflect the usual human obesity condition, in which most obese humans are leptin resistant. However, this model does allow us to examine the influence of exogenous leptin on gene expression and therefore provides a framework for further studies.

In conclusion, the current study identified a subset of genes regulated by leptin and involved in absorption and/or secretion in the gallbladder. These genes provide insight into the role absorption and/or secretion may play in gallstone pathogenesis. Moreover, the results of this study, although interesting, suggest that alterations in sodium- and water-transport processes may be regulated differently in the setting of obesity. Such results provide a framework for future physiological studies to investigate pathophysiological processes.

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