Hepatic Insulin Resistance Directly Promotes Formation of Cholesterol Gallstones

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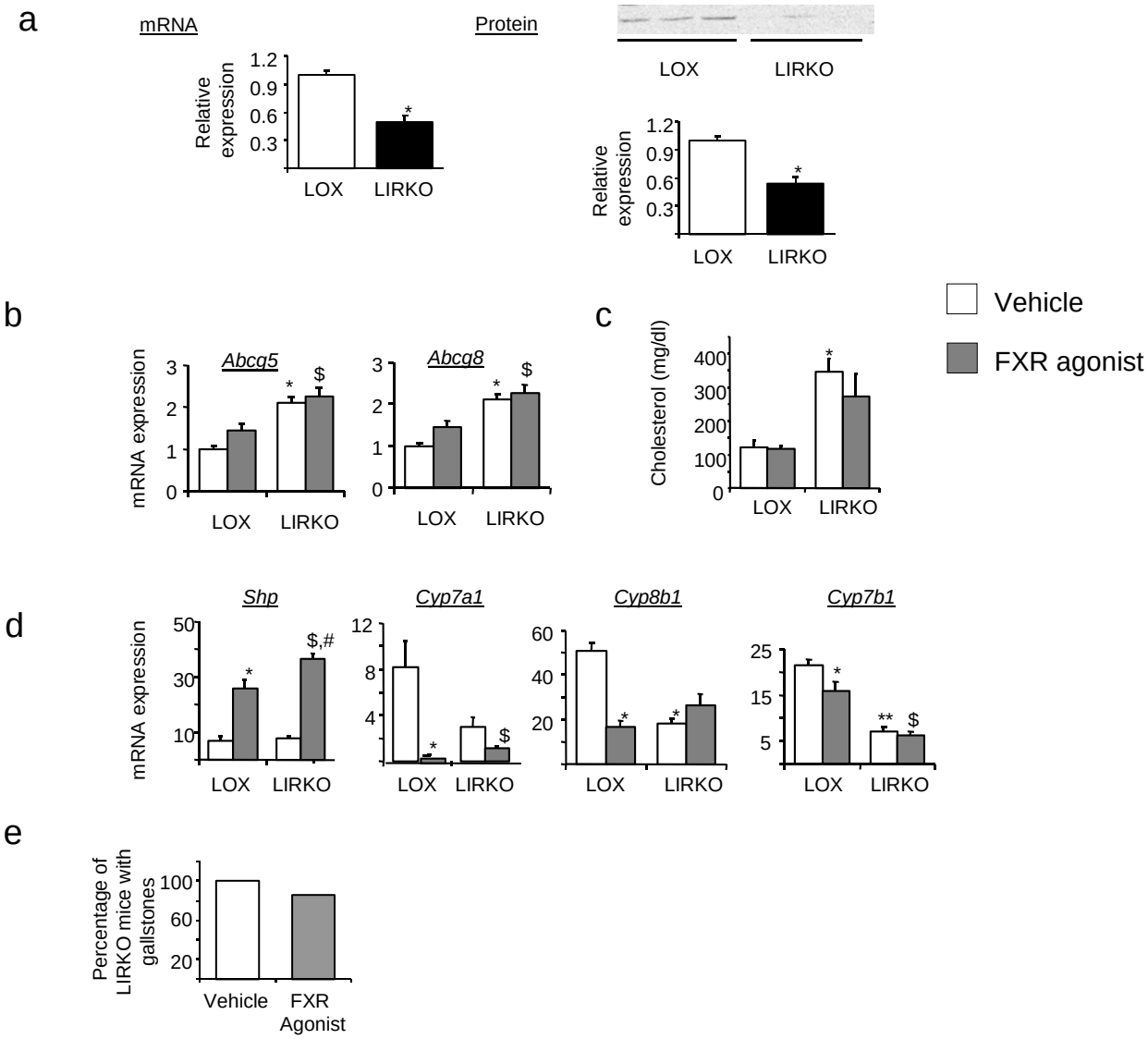

Supplemental Figure 1. *LIRKO mice are resistant to an FXR agonist.* (a) Expression of FXR using real time PCR analysis and immunoblotting of nuclear extracts prepared from the livers of non-fasted three month old mice. Quantitation of the immunoblots by scanning densitometry is shown below. (b-d) Two-month old mice were gavaged with an FXR agonist (GW4064) at 100 mg/kg/d or vehicle for 14 days. Mice were fasted overnight, and sacrificed two hours after the last dose of agonist. Bile was expressed from the gallbladders. (b,d) Real time PCR was performed on cDNA prepared from these livers. (c) Biliary cholesterol content was quantitated using high performance liquid chromatography (n=4-8). (e) Three month old LIRKO mice were placed on the lithogenic diet for three weeks. During that those three weeks, they were gavaged with an FXR agonist (GW4064) at 100 mg/kg/d. At the conclusion of the experiment, mice were sacrificed, and their gallbladders were examined for the presence of stones (n=7-8) *$p<0.05$ versus Lox untreated; $^{\#}p<0.05$ versus LIRKO untreated, $^{\$}p<0.05$ versus Lox treated.

Supplementary Tables

	Mol%CH	Mol%PL	Mol%BS	TLC (g/dL)	CSI
Hepatic Bile					
LOX	**2.56 ± 0.33**	**10.8 ± 0.99**	**86.6 ± 1.32**	**3.05 ± 0.53**	**1.08 ± 0.13**
LIRKO	**6.82 ± 0.36****	**14.1 ± 1.4**	**79.1 ± 1.5***	**2.80 ± 0.36**	**1.92 ± 0.15****
Gallbladder Bile					
LOX	**1.71 ± 0.22**	**10.4 ± 0.8**	**87.9 ± 1.0**	**9.69 ± 1.56**	**0.53 ± 0.07**
LIRKO	**3.98 ± 0.46****	**13.0 ± 0.4***	**83.0 ± 0.6***	**11.43 ± 0.55**	**0.85 ± 0.10***

Supplementary Table 1. *Biliary Lipid Compositions of Hepatic and Gallbladder Bile Specimens.* Cholesterol (CH), phospholipid (PL) and bile salt (BS) concentrations were measured as described in methods, and divided by the total molar lipid concentration to obtain the relative lipid concentration (mol%). The total lipid concentration (TLC) was calculated as the sum of the CH, PL and BS concentrations. Cholesterol saturation indices (CSIs) were calculated from critical tables. *$p \leq 0.05$, **$p \leq 0.005$ compared to Lox.

	LIRKO	HFD	Genetic Back-ground	Leptin Deficiency
Cyp7b1	0.17*	0.55*	0.36*	0.49
Cyp7a1	0.72	0.74	0.89	0.42
Cyp8b1	0.49*	0.83	1.53*	1.54

Supplementary Table 2. *Bile acid synthetic enzyme expression in models of insulin resistance*. LIRKO mice were compared to their littermate controls (LOX), 129Sv mice were fed either a high fat (HFD) or low fat (LFD), or mice with an insulin resistant genetic background (C57Bl/6 strain) were compared to normal mice (129 strain)[1], and *ob/ob* mice were compared to their lean littermates. Data are presented as the ratio of the mean expressions in each group (n=3-4 chips per group, with RNA from 2-3 mice pooled for each chip). Blue and red indicate a decrease, or increase, respectively, of gene expression by more than 20%. * $p<0.05$.

	LIRKO	Diet Induced Obesity
ABCG5	**2.50***	**1.35***
ABCG8	**2.10***	**1.28***
Cyp7b1	**0.32***	**0.43***
Cyp7a1	**0.12***	**0.40***
Cyp27a1	**0.74***	**0.96**
Cyp8b1	**0.54***	**1.19**

Supplementary Table 3. Gene Expression in mice with diet induced obesity using real time PCR. C57Bl/6 mice were placed on a high (55% calories from fat) or low (12% calories from fat) fat diet for 18 weeks, at which time they were sacrificed in the non-fasted stated [1]. Gene expression was measured by real time PCR. Data are expressed as a fold change (High fat diet/Low fat diet), and *$p<0.05$ (n=6-8). Values for LIRKO mice taken from Figures 1 and 2 are shown for comparison.

Supplementary Methods

Biliary and Hepatic Lipid Studies. After caudad ligation of the common bile duct and ligation of the cystic duct, we cannulated the common bile duct with a polyethylene catheter (PE-10). Hepatic bile was collected under gravity drainage for 30 to 60 minutes. Gallbladder bile was obtained following cholecystectomy by expressing digitally the contents of the gallbladder. We determined bile salt species by high performance liquid chromatography (HPLC) followed by comparing retention times with bile salt standards[2]. Gallbladder bile obtained from FXR-agonist treated mice showed some peaks attributable to the agonist itself, but represented less than 10% of the total bile acids. The hydrophobicity index was calculated using the method of Heuman[3], assuming equal amounts of tauroα- and tauroβ-muricholates. Biliary phospholipid concentrations were determined by the inorganic phosphorus method[4]. Biliary cholesterol was measured by HPLC after extraction with hexane[5]. We measured total bile salt concentrations by a spectrophotometric assay based on 3α-hydroxysteroid dehydrogenase[6]. Secretion rates in hepatic bile were obtained by multiplying bile flow rates by biliary lipid concentrations. CSI values were calculated from the critical tables[7], after correcting for the presence of muricholates with an "ursodeoxycholate" correction factor[7,8]. We calculated the relative molar proportions of cholesterol, bile salts and phospholipids from their absolute biliary concentrations, and plotted the results on a partial condensed phase diagram drawn for a total lipid content of 10 g/dL, as a 2.5g/dL diagram simulating dilute hepatic bile is not available[9]. Hepatic cholesterol was measured by gas chromatography as previously described[10] or using an enzymatic assay (Wako Chemicals).

Fecal Bile Acids. Feces were collected from individually housed mice over three days and analyzed as described[11]. Quantitation was performed by the use of 3α-hydroxysteroid dehydrogenase.

Cholesterol Absorption. We measured cholesterol absorption by the fecal dual isotope ratio method [12]. After a six-hour fast, we gavaged mice with a mixture of 1 μCi of [4-^{14}C]cholesterol and 2 μCi of [5,6-^{3}H]β -sitostanol in medium-chain triglyceride oil. Stool was collected over the next 72 hours, lyophilized, and weighed. Equal aliquots of stool were bleached with hydrogen peroxide and subjected to scintillation counting. The absorption was calculated as follows:

$$\frac{\left(^{14}C/_{3}H\right)_{gavage} - \left(^{14}C/_{3}H\right)_{excreted}}{\left(^{14}C/_{3}H\right)_{gavage}} \text{ x } 100\%.$$

Affymetrix Analysis. We prepared RNA from the livers of the following male mice: 6-8 week old LIRKO and their controls, sacrificed in the non-fasted state; 24-week old 129 or B6 mice purchased from Taconic, fed a low fat (LFD, 14%, 25% and 61% calories from fat, protein and carbohydrate respectively) or high fat (HFD, 55%, 21% and 24% calories from fat, protein and carbohydrate respectively) diet for 18 weeks, and sacrificed in the non-fasted state; four month old ob/ob mice and their controls purchased from Jackson Labs, and sacrificed after a 3-4 hour fast. We pooled RNA from 2-3 livers for each chip, and analyzed 3-4 chips (Affymetrix, Mu74av2) per group, using GENECHIP MAS v.5.0.

Supplementary References

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