Hepatic insulin receptor deficiency impairs the SREBP-2 response to feeding and statins[S]

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Abstract **The liver plays a central role in metabolism and mediating insulin action. To dissect the effects of insulin on the liver in vivo, we have studied liver insulin receptor knockout (LIRKO) mice. Because LIRKO livers lack insulin receptors, they are unable to respond to insulin. Surprisingly, the most profound derangement observed in LIRKO livers by microarray analysis is a suppression of the cholesterologenic genes. Sterol regulatory element binding protein (SREBP)-2 promotes cholesterologenic gene transcription, and is inhibited by intracellular cholesterol. LIRKO livers show a slight increase in hepatic cholesterol, a 40% decrease in *Srebp-2*, and a 50–90% decrease in the cholesterologenic genes at the mRNA and protein levels. In control mice, SREBP-2 and cholesterologenic gene expression are suppressed by fasting and restored by refeeding; in LIRKO mice, this response is abolished. Similarly, the ability of statins to induce *Srebp-2* and the cholesterologenic genes is lost in LIRKO livers. In contrast, ezetimibe treatment robustly induces *Srepb-2* and its targets in LIRKO livers, raising the possibility that insulin may regulate SREBP-2 indirectly, by altering the accumulation or distribution of cholesterol within the hepatocyte. Taken together, these data indicate that cholesterol synthesis is a key target of insulin action in the liver.**—Miao, J., J. T. Haas, P. Manthena, Y. Wang, E. Zhao, B. Vaitheesvaran, I. J. Kurland, and S. B. Biddinger. **Hepatic insulin receptor deficiency impairs the SREBP-2 response to feeding and statins. *J. Lipid Res*. 2014.** 55: **659–667.**

Supplementary key words hepatic insulin signaling • cholesterol biosynthesis • sterol regulatory element binding protein • liver insulin receptor knockout

Diabetes affects almost 26 million Americans but the mechanisms by which it promotes CVD, the most common cause of death in diabetic patients (1, 2), and other complications, such as nonalcoholic fatty liver disease (NAFLD), remain unclear. Both type 1 and type 2 diabetes arise from defects in insulin action: type 1 diabetes is caused by autoimmune-mediated destruction of the β-cells of the pancreas, resulting in insulin deficiency, whereas type 2 diabetes is caused by insulin resistance and decompensation of the β-cells (3). Though the most salient action of insulin is to prevent hyperglycemia, the development of CVD and NAFLD are not fully prevented by the normalization of blood glucose levels (4). Thus, insulin actions on pathways distinct from glucose metabolism may play a role in the development of CVD, NAFLD, and other complications of diabetes.

Derangements in cholesterol metabolism are closely associated with atherosclerosis, and could also contribute to the development of NAFLD (5). Cholesterol biosynthesis and its regulation have therefore been intensively studied over many years (6). For example, the regulation of cholesterol synthesis by dietary cholesterol has been found to be mediated in large part by the transcription factor sterol regulatory element binding protein (SREBP)-2. SREBP-2 is synthesized as a membrane bound precursor which must undergo proteolytic cleavage to generate its active nuclear form (7). This cleavage is inhibited by intracellular cholesterol. When intracellular cholesterol levels are low, SREBP-2 increases transcription of the cholesterologenic enzymes and the LDL receptor (LDLR), resulting in increased intracellular cholesterol via a combination of increased cholesterol synthesis and uptake. When cholesterol levels are high, SREBP-2 processing is inhibited, preventing synthesis and uptake of cholesterol. Similarly, statin drugs, which inhibit HMG-CoA reductase (HMGCR) and cholesterol synthesis, are thought to activate SREBP-2 by reducing the amount of cholesterol in the regulatory pool of the cell.

Abbreviations: FDPS, farnesyl diphosphate synthase; HMGCR, HMG-CoA reductase; LDLR, LDL receptor; LIRKO, liver insulin receptor knockout; NAFLD, nonalcoholic fatty liver disease; SREBP, sterol regulatory element binding protein.

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However, dietary cholesterol and cholesterol lowering drugs are not the only factors which regulate cholesterol synthesis. Indeed, over 50 years ago it was shown that fasting decreases cholesterol synthesis, whereas refeeding induces it (8, 9). Since then, SREBP-2 and the mRNA levels of the cholesterologenic enzymes have been shown to decrease with fasting and normalize with refeeding (10); these changes are concurrent with changes in plasma insulin levels, which also fall with fasting and rise with refeeding.

To define the role of insulin in the liver, a key site of insulin and statin action and metabolism, we have used liver insulin receptor knockout (LIRKO) mice. LIRKO mice are homozygous for the floxed allele of the insulin receptor, and heterozygous for the Cre transgene expressed under the albumin promoter. Consequently, LIRKO hepatocytes lack the insulin receptor and insulin signaling (11). As expected, LIRKO mice are mildly hyperglycemic, and fail to suppress hepatic glucose production in response to insulin (12).

Here, we show that LIRKO mice manifest decreased nuclear SREBP-2 protein, decreased cholesterologenic gene expression, and decreased cholesterol synthesis. The normal response of SREBP-2 and its targets to fasting and feeding is abolished in LIRKO mice. Similarly, statin drugs are unable to induce SREBP-2 and its targets in LIRKO livers. On the other hand, ezetimibe, alone or in conjunction with statin drugs, is effective in inducing SREBP-2 and its targets in LIRKO mice.

MATERIALS AND METHODS

Animals, diets, and treatments

Generation and genotyping of LIRKO ($Cre^{+/-}$, $IR^{lox/lox}$) mice and their littermate Lox controls ($Cre^{-/-}$, $IR^{lox/lox}$) have been described previously (11). LIRKO mice were maintained on a mixed genetic background, including 129/Sv, C57BL/6, FVB, and DBA, and inbred for more than thirty generations. Mice were housed with a 12 h light cycle (7:00 AM to 7:00 PM) and fed a standard rodent chow. For fasting and refeeding studies, mice were euthanized at 2:00 PM under the following conditions: ad libitum fed, after a 24 h fast, or after a 24 h fast followed by refeeding a high carbohydrate diet for 6 h (TD 88122, Harlan Teklad Diets). For the lovastatin and lovastatin/ezetimibe treatments, mice were given free access to powdered chow, or powdered chow supplemented with 0.1% lovastatin (w/w), 0.025% ezetimibe (w/w), or both. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children's Hospital Boston.

Microarray experiments

Affymetrix MU74Av2 arrays (n = 3 per group) were used to measure transcripts from control and LIRKO livers. Total RNA (RNeasy, Qiagen) was isolated from the livers of nonfasted mice (RNA from 2–3 mice was pooled for each chip) and prepared for hybridization per the manufacturer's instructions. Raw data was processed in R using the open-source Bioconductor packages, affy (13) and limma (14). Samples were background corrected and normalized using robust multichip averaging (13). Expression values were extracted and gene set enrichment analysis (15, 16) was performed using the most current GenMAPP annotation (17).

Gene expression analysis

RNA was isolated and 2 μg were used for reverse transcription (Applied Biosystems, Foster City, CA). The resultant cDNA was diluted at least 5-fold and used for real-time PCR analysis with SYBR green reagents (Fermentas). Results were normalized to 18S expression. Primer sequences are listed in supplementary Table II.

Phenotypic and biochemical characterization

Plasma was obtained using EDTA-treated syringes and colorimetric assays were used for measuring total cholesterol (Wako Chemicals and Thermo Scientific) and total triglycerides (Thermo Scientific). Hepatic lipids were extracted in the method of Folch (18). Briefly, 50 mg of liver was homogenized in 50 mM NaCl and extracted with chloroform:methanol (2:1). The interphase was washed once with 50 mM NaCl and once with 0.36 M $CaCl_2$ in 50% methanol. Aliquots of the organic extract were supplemented with Triton X-100 (Sigma) and dried under a nitrogen evaporator at room temperature. Colorimetric reagents for measuring total cholesterol were added directly to the detergent pellet and read per the manufacturer's instructions. Fast protein liquid chromatography fractionation was performed by the Vanderbilt Mouse Metabolic Phenotyping Center.

Western blotting

Liver lysates were prepared by homogenizing 50 mg of liver in 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.3% SDS, 10 mM NaF, 10 mM Na_3VO_4, 10 mM sodium β-glycerophosphate, and protease inhibitor (Roche) and centrifuging at 13,000 *g* for 10 min at 4°C. Protein was measured by the BCA assay kit (Pierce). The sample (100 μg) was loaded on SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane (Immobilon). After 1 h in SuperBlock blocking buffer (Pierce), blots were incubated overnight with the following antibodies diluted in SuperBlock blocking buffer: HMGCR (sc-27578; Santa Cruz Biotechnology), IDI1 (ab81491; Abcam), farnesyl diphosphate synthase (FDPS) (ab38854; Abcam), SQLE (12544-1-AP; Labome), CYP51 (H00001595-A01; Novus Biologicals), INSR (sc-711; Santa Cruz Biotechnology), β-actin (sc-47778; Santa Cruz Biotechnology), and α-tubulin (sc-8035; Santa Cruz Biotechnology). LDLR antibodies were a kind gift from Dr. Attie (19). Nuclear and membrane extracts were prepared and subjected to immunoblotting as previously described (20); SREBP-2 antibodies were a kind gift from Dr. Horton (21); antibodies against lamin A/C (2032; Cell Signaling) and calnexin (sc-6465; Santa Cruz Biotechnology) were purchased commercially. Secondary antibody conjugated with horseradish peroxidase (Pierce) and chemiluminescent ECL reagents (Pierce) were used to develop blots.

De novo cholesterologenesis

Cholesterologenesis was measured as previously described (23) using gas chromatography-electron impact ionization mass spectrometry (22) with the following modifications: the elution time of cholesterol was ~10.7 min and ^2H-enrichment was determined using selective ion monitoring under electron impact ionization of *m/z* 368 and 369 (M+0 and M+1) for cholesterol. Cholesterologenesis was calculated according to the following formula, where n is the number of exchangeable hydrogens and assumed to equal 25: percent newly synthesized cholesterol = 100 × [total ^2H-labeling cholesterol/(^2H-labeling body water/n)].

RESULTS

Microarray analysis

We used oligonucleotide microarrays to query, in a nonbiased manner, the effects of hepatic insulin signaling on

gene expression. Gene set enrichment analysis revealed cholesterol biosynthesis to be the pathway most significantly altered in LIRKO livers (**Fig. 1A**). Gluconeogenesis and glycolysis, which are classic targets of insulin action, were also found by gene set enrichment analysis to be significantly changed (supplementary Table I). However, the cholesterol biosynthetic pathway was surprisingly ranked higher. A heat map of the 14 cholesterologenic genes represented on the array showed these genes to be coordinately decreased in LIRKO livers (Fig. 1B).

The decrease in cholesterologenic gene expression was also observed at the protein level: HMGCR, the rate determining enzyme of cholesterol synthesis, and all other cholesterologenic enzymes examined were decreased 80–90%. Insulin receptor (INSR) protein was decreased by more than 90% (Fig. 1C). Taken together, these data indicate that, at the level of gene expression, cholesterol biosynthesis is a key target of insulin action in the liver.

Role of insulin in the control of cholesterol synthesis by fasting and feeding

The transition between fasting and feeding is coordinated by numerous hormones, as well as nutrients themselves. To specifically dissect the role of insulin in this transition, we examined the effects of fasting and feeding on cholesterol synthesis in LIRKO mice. The livers of LIRKO mice are unable to respond to insulin, but still capable of responding to nutrients and other hormones (23, 24).

In control mice, 24 h of fasting decreased *Srebp-2* mRNA levels by 50% and nuclear protein levels by more than 80%; refeeding restored these levels toward normal (**Fig. 2A, B**), consistent with prior reports (10, 25). In LIRKO livers, *Srebp-2* mRNA was decreased by 40% in the nonfasted state, and did not change with either fasting or refeeding. Nuclear SREBP-2 protein was markedly decreased in LIRKO livers and appeared unresponsive to fasting or refeeding (Fig. 2A, B).

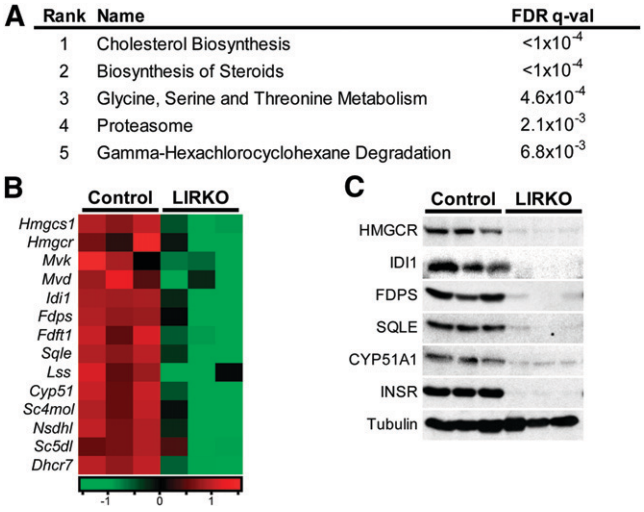

Fig. 1. Cholesterologenic gene expression is decreased in the livers of LIRKO mice. LIRKO mice and their littermate controls were euthanized in the nonfasted state at 2–3 months of age. A, B: RNA was isolated and subjected to microarray analysis. A: Data were analyzed using gene set enrichment analysis. The five top ranking gene sets and associated False Discovery Rate q-values, estimations of the significance adjusted for multiple hypotheses testing, are listed. B: A heatmap showing the 14 genes represented in the cholesterol biosynthesis gene set. C: Immunoblots were performed on liver extracts prepared from 8–10-week-old mice euthanized in the nonfasted state.

SREBP-2 is a member of the family of SREBP proteins, which are encoded by two genes. *Srebp-1* is alternatively spliced to produce *Srebp-1a* and *Srebp-1c*. *Srebp-1a* is expressed at low levels in the liver, and can activate transcription of both the cholesterologenic and lipogenic enzymes (26). In contrast, *Srebp-1c* is the predominant *Srebp-1* isoform in the liver (27). SREBP-1c is induced by insulin and controls primarily the lipogenic enzymes. In control mice, both *Srebp-1a* and *Srebp-1c* were reduced by fasting and induced by refeeding, though *Srebp-1c* was much more sensitive to these manipulations (Fig. 2C, D). In LIRKO livers, *Srebp-1a* levels were similar to controls, and did not respond significantly to fasting or refeeding; *Srebp-1c* levels were decreased in LIRKO livers, and the response to fasting and refeeding was present but blunted (Fig. 2C, D). In parallel, nuclear SREBP-1 protein and lipogenic gene expression were decreased in LIRKO livers, particularly in the refed state (28). Thus, both SREBP-1 and SREBP-2 expression were compromised in LIRKO livers.

Real-time PCR analysis of the livers of control mice showed that mRNA levels of the cholesterologenic enzymes, *Hmgcr*, farnesyl diphosphate synthase (*Fdps*), and *Cyp51*, fell by 70–90% after a 24 h fast. When mice were refed a high carbohydrate diet for 6 h, the expression of these genes increased toward nonfasted levels (Fig. 2E–G). In the livers of nonfasted LIRKO mice, *Hmgcr*, *Fdps*, and *Cyp51* mRNA levels were reduced 40–90% relative to controls (Fig. 2E–G), confirming our microarray data (Fig. 1B), and showed little change with fasting or refeeding. Finally, the induction of cholesterol synthesis was markedly blunted, such that cholesterol synthesis was only 20% of controls after 48 h of refeeding (Fig. 2H).

Plasma cholesterol levels were decreased in LIRKO mice compared with controls (Fig. 2I). This was consistent with our prior work showing that HDL cholesterol, the largest component of plasma cholesterol in the mouse, is decreased in LIRKO mice on the chow diet (28). On the other hand, hepatic cholesterol levels were slightly, but significantly, elevated in LIRKO mice in the basal state (Fig. 2J), despite decreased cholesterol synthesis. Fasting tended to increase cholesterol accumulation in both control and LIRKO livers.

Statins fail to induce *Srebp-2* and its targets in LIRKO livers

Statin drugs are important therapeutic agents which activate SREBP-2 by inhibiting cholesterol synthesis. To determine the effects of insulin on the statin response, LIRKO mice and their controls were treated with statins (0.1% lovastatin) for 1 week. Statin treatment did not significantly change hepatic cholesterol in either control or LIRKO livers (**Fig. 3A**). Nonetheless, in control livers, statin treatment increased mRNA levels of *Srebp-2* by 50% and the cholesterologenic genes *Hmgcr*, *Fdps*, and squalene

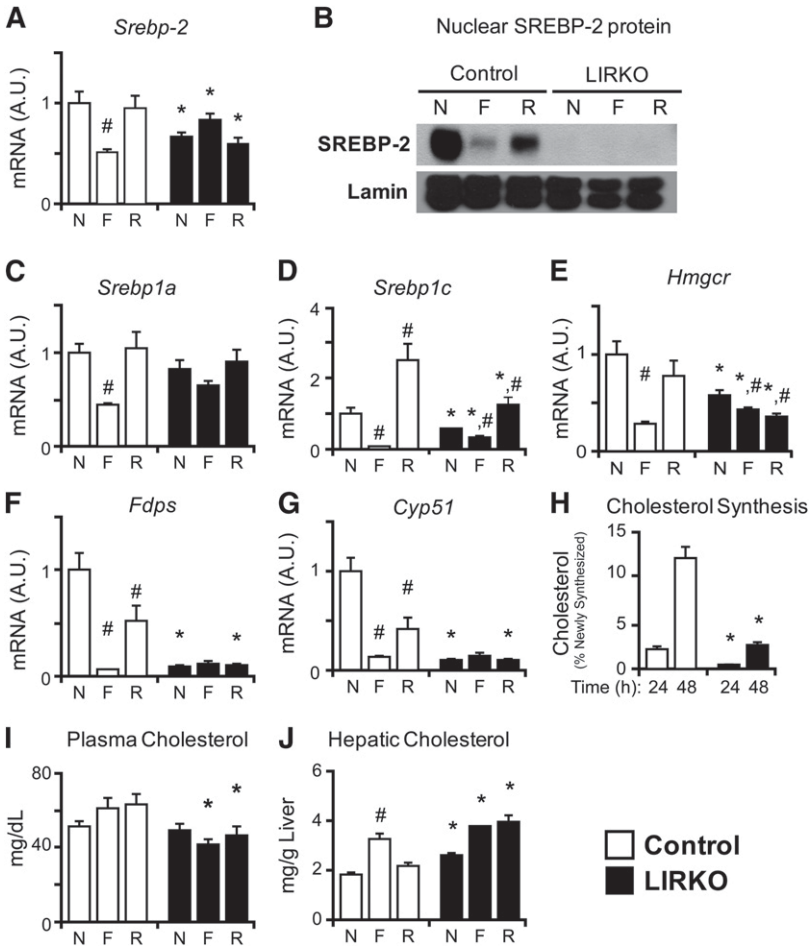

Fig. 2. The fasting/feeding response of the cholesterologenic genes is blunted in LIRKO livers. LIRKO mice and their littermate controls were euthanized either in the nonfasted state (N), after a 24 h fast (F), or after a 24 h fast with subsequent refeeding of a high carbohydrate diet for 6 h (R). A, C–G: Gene expression was measured using real-time PCR. B: SREBP-2 protein levels were measured in nuclear extracts prepared from the livers of these mice. Each lane represents a pool prepared from equal aliquots of three livers. H: De novo cholesterol synthesis was measured as the fraction of newly synthesized cholesterol present 24 or 48 h after refeeding. I, J: Cholesterol levels were measured in plasma and liver taken at the time of euthanasia. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same feeding conditions; $^{\#}P < 0.05$ versus nonfasted mice of the same genotype.)

synthase (*Fdft1*) by 2- to 4-fold (Fig. 3B–E). In contrast, in LIRKO livers, statin treatment failed to induce the cholesterologenic enzymes.

PCSK9 (29) and IDOL (30) are two proteins which promote the degradation of the LDLR. *Pcsk9* is paradoxically induced by SREBP-2 (29), whereas *Idol* is induced by oxysterols via the transcription factor liver X receptor (30). In control mice, statin treatment increased both *Ldlr* and *Pcsk9* mRNA, as expected given the induction of SREBP-2 (Fig. 3F, G). *Idol* was not changed (Fig. 3H). LDLR protein, presumably because of the induction of *Pcsk9*, was slightly reduced in statin-treated control mice (Fig. 3I). In the absence of treatment, LIRKO livers showed decreased *Ldlr* and *Pcsk9* mRNA, increased *Idol* mRNA, and normal levels of LDLR protein relative to control livers (Fig. 3F–I). Note that this is in contrast to the lower levels of LDLR protein observed in LIRKO versus control mice on the high cholesterol cholic acid-containing Paigen diet (28), reflecting an interaction of diet and insulin in the control of LDLR protein. In response to statin treatment, LIRKO livers showed no change in *Idol* or *Pcsk9* expression, but LDLR mRNA and protein levels were decreased (Fig. 3F–I).

Plasma triglycerides and cholesterol fell with statin treatment in control mice, though the results did not reach significance (Fig. 3J, K). In contrast, statin treatment did not change triglyceride levels in LIRKO mice, and increased plasma cholesterol levels by more than 50% (Fig. 3J, K). Thus, the inhibition of cholesterol synthesis is not sufficient to induce *Srebp-2* or its targets in LIRKO livers.

Lovastatin/ezetimibe combination therapy reverses changes in SREBP-2 and cholesterologenic gene expression in LIRKO livers

Another drug used to lower serum cholesterol is ezetimibe, which blocks the absorption of cholesterol from the gut by inhibiting the cholesterol transporter NPC1L1 (31, 32). Ezetimibe (0.25%) was fed to control and LIRKO mice for 1 week. Though we were unable to detect consistent effects of ezetimibe on hepatic cholesterol, ezetimibe was clearly able to induce the cholesterologenic genes in both control and LIRKO mice (**Fig. 4A–F**). Thus, ezetimibe treatment increased *Srebp-2* in LIRKO livers, and increased the cholesterologenic genes, *Hmgcr*, *Fdps*, *Fdft1*, and *Cyp51* in the livers of both LIRKO and control mice (Fig. 4B–F). Also, ezetimibe treatment increased *Ldlr* mRNA in control livers and slightly but significantly reduced plasma cholesterol in LIRKO mice (Fig. 4G, H).

A combination of statin and ezetimibe (0.1% lovastatin/0.025% ezetimibe, for 1 week) had an even more profound effect on SREBP-2 in LIRKO livers (31). Statin/ezetimibe treatment decreased hepatic cholesterol levels and increased *Srebp-2* mRNA in both control and LIRKO mice (**Fig. 5A, B**). SREBP-2 precursor protein, which was

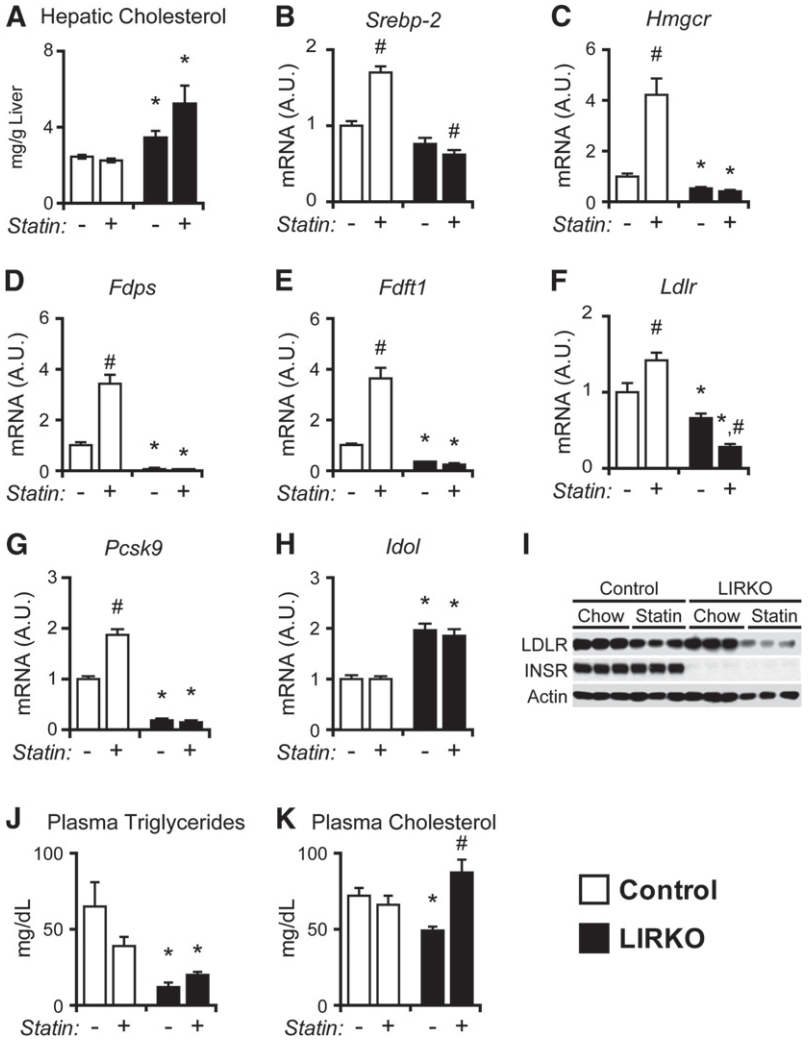

Fig. 3. The response to statins is abolished in LIRKO livers. LIRKO and control mice were fed a standard chow diet with or without supplementation of 0.1% lovastatin for 1 week and euthanized in the nonfasted state. A: Hepatic cholesterol was measured using a colorimetric assay. B–H: Gene expression was measured using real-time PCR. I: LDLR protein was measured in liver homogenates by immunoblotting. J, K: After 5 days on the chow diet with or without lovastatin supplementation, mice were fasted for 4 h, and plasma obtained for the measurement of triglycerides and cholesterol. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same treatment conditions; $^{\#}P < 0.05$ versus untreated mice of the same genotype.)

difficult to detect under basal conditions, was markedly induced by statin/ezetimibe treatment, as was nuclear SREBP-2 protein (Fig. 5C). The effects of statin/ezetimibe treatment were most remarkable in LIRKO livers, raising *Srebp-2* mRNA and protein to levels even higher than similarly treated controls. In parallel, statin/ezetimibe treatment produced 5- to 50-fold inductions in the cholesterologenic enzymes (Fig. 5D–G).

The processing of SREBP-2 is inhibited by the INSIG proteins, INSIG1 and INSIG2. The SREBPs induce transcription of *Insig1* as part of an inhibitory feedback loop (25). Thus, SREBP activation promotes the accumulation of INSIG1, which inhibits further SREBP processing. *Insig1* mRNA was decreased in LIRKO versus control livers in the basal state, consistent with the lower SREBP levels, and induced in mice of both genotypes by statin/ezetimibe treatment (Fig. 5H). *Insig2* is encoded by two transcripts, *Insig2a* which is suppressed by feeding and induced by streptozotocin, which renders mice insulin deficient, and *Insig2b,* a ubiquitous transcript (33). In the basal state, LIRKO livers showed a decrease in *Insig2a* and an increase in *Insig2b* (Fig. 5I, J), consistent with prior reports (23). Neither *Insig2a* nor *Insig2b* was consistently changed in either control or LIRKO livers by statin/ezetimibe treatment.

Statin/ezetimibe treatment increased *Ldlr* and *Pcsk9* mRNA levels in both control and LIRKO mice and tended to decrease *Idol* mRNA in LIRKO mice **(Fig. 6A–C)**. Though consistent changes in LDLR protein were not observed (Fig. 6D), statin/ezetimibe treatment decreased plasma cholesterol by approximately 15% in control mice and more than 70% in LIRKO mice (Fig. 6E). This decrease was due to a lowering of HDL in both control and LIRKO mice, consistent with prior studies in mice (34–36), but also to a decrease in LDL/IDL in LIRKO mice (supplementary Fig. I).

Interestingly, the effects of cholesterol depletion on *Srebp-1c* and the lipogenic enzymes were also intact in LIRKO livers. Previous studies have shown that the depletion of hepatic cholesterol by either bile acid sequestrants (20) or ezetimibe (31) in combination with statin drugs leads to a decrease in nuclear SREBP-1 protein but an increase in SREBP-1 and SREBP-2 binding to the lipogenic gene promoters. Consequently, lipogenic gene expression increases upon cholesterol depletion (31). Here, as expected, statin/ezetimibe treatment decreased *Srebp-1c* mRNA levels by 50% but induced *Fasn* and stearoyl-CoA desaturase 1 (*Scd1*) 5- to 10-fold in control mice (**Fig. 7A–C**). Statin/ezetimibe treatment had the same

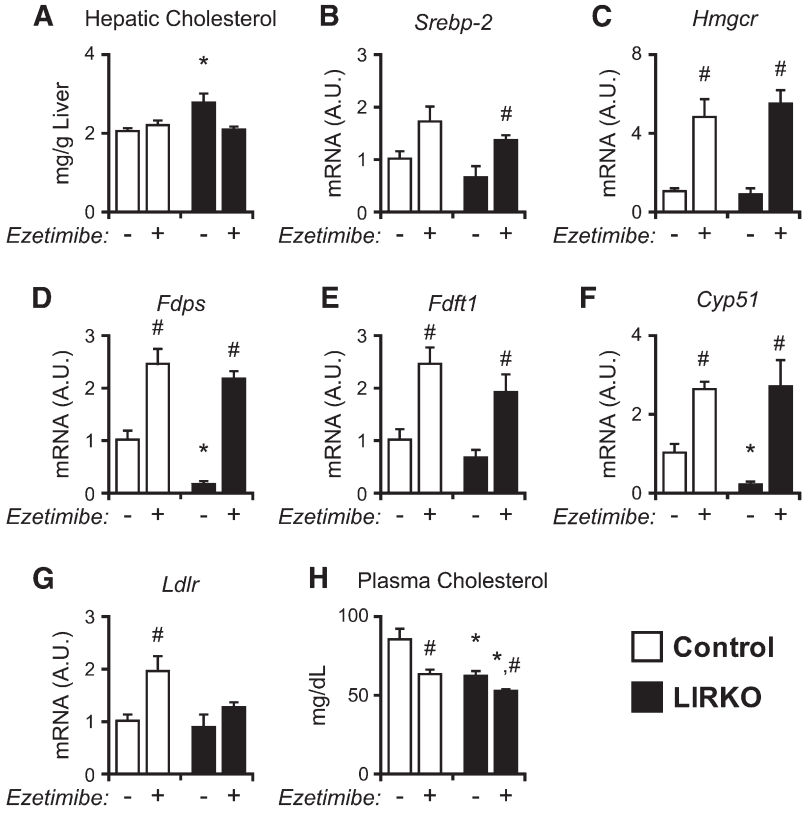

Fig. 4. Treatment with ezetimibe only is sufficient to induce SREBP-2 and the cholesterologenic genes in LIRKO livers. LIRKO and control mice were fed a diet with or without supplementation with 0.025% ezetimibe for 1 week and were euthanized in the nonfasted state. A: Cholesterol was measured in the livers of these mice using a colorimetric assay. B–G: Gene expression was measured using real-time PCR. H: Cholesterol was measured in the plasma of nonfasted mice at the time of euthanasia. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same treatment conditions; $^{\#}P < 0.05$ versus untreated mice of the same genotype.)

effects in LIRKO mice: *SREBP-1c* was decreased by more than 50%, but *Fasn* and *Scd1* were increased by 10- and 4-fold, respectively. In parallel with lipogenic gene expression, hepatic triglycerides were increased by statin/ezetimibe treatment in both control and LIRKO livers (Fig. 7D). However, plasma triglycerides were reduced 40% in control mice by statin/ezetimibe treatment (Fig. 7E). In LIRKO mice, plasma triglycerides were unchanged by statin/ezetimibe treatment, perhaps because VLDL triglyceride secretion, which is low in untreated LIRKO mice (23), was normalized by the increase in lipogenic gene expression and hepatic triglycerides.

DISCUSSION

Insulin is a key anabolic hormone with pleiotropic effects. Surprisingly, of all the changes in gene expression produced by the ablation of hepatic insulin signaling, the most pervasive and profound change occurs in the cholesterol biosynthesis gene set. LIRKO livers show reduced SREBP-2, cholesterologenic gene expression, and cholesterol synthesis. Moreover, the ability of fasting to decrease cholesterologenic gene expression and refeeding to restore it is almost abolished in LIRKO livers. Yet, the ability of statin/ezetimibe treatment to induce SREBP-2 and its targets is not compromised in LIRKO livers.

Both fasting and knockout of the insulin receptor reduce insulin signaling, SREBP-2, cholesterologenic gene expression, and cholesterol synthesis. Mice with defects in hepatic insulin signaling due to knockout of insulin receptor substrates, IRS-1 and IRS-2, which mediate most of insulin's actions on the hepatocyte, also show decreased expression of SREBP-2 and the cholesterologenic enzymes (37, 38). Taken together, these data indicate that insulin either directly or indirectly induces SREBP-2 and the cholesterologenic enzymes.

The mechanisms by which insulin induces SREBP-2 and its targets remain under investigation, but there are several possibilities. First, mTORC1, which induces nuclear SREBP-2, is a downstream target of insulin (39). Though mTORC1 can be activated by nutrients even in LIRKO livers (23), it is possible that LIRKO mice have an inability to fully activate mTORC1, leading to reduced SREBP-2 levels. Second, the decrease in cholesterologenic gene expression observed in the absence of insulin signaling could be due to decreased levels of SREBP-1c, which also binds the promoters of the cholesterologenic genes (31). Third, insulin could indirectly regulate SREBP-2 by altering the amount of cholesterol in the regulatory pool, presumably the free cholesterol in the endoplasmic reticulum membrane. In support of this notion, bile acid synthesis is decreased in LIRKO mice (40) and treatment with ezetimibe alone induced *Srebp-2* and its targets in LIRKO mice. Thus, decreased conversion of cholesterol to bile acids, and increased cholesterol delivery to the hepatocyte from the gut could promote cholesterol accumulation in the LIRKO liver and suppress SREBP-2. However, we were unable to detect any differences in the amount of free cholesterol in the livers of control and LIRKO mice (data not shown). Nonetheless, it is possible that insulin alters the

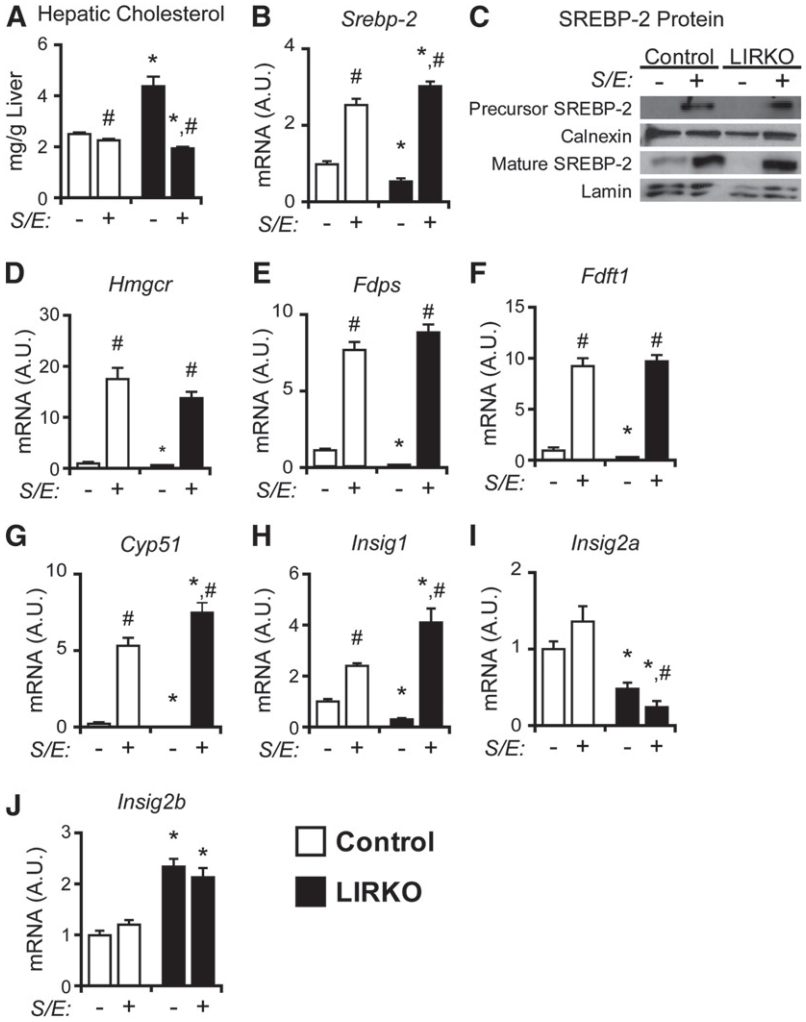

Fig. 5. Statin/ezetimibe treatment induces SREBP-2 and the cholesterologenic genes in LIRKO livers. LIRKO and control mice were fed a diet with or without supplementation with statin/ezetimibe (S/E) (0.1% lovastatin and 0.025% ezetimibe) for 1 week and were euthanized in the nonfasted state. A: Cholesterol was measured in the livers of these mice using a colorimetric assay. B, D–J: Gene expression was measured using real-time PCR. C: SREBP-2 protein levels were measured in microsomal extracts (Precursor) or nuclear extracts (Mature) prepared from the livers of these mice. Each lane represents a pool prepared from equal aliquots of three livers. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same treatment conditions; $^{\#}P < 0.05$ versus untreated mice of the same genotype.)

subcellular distribution of cholesterol, such that the free cholesterol content of the endoplasmic reticulum membrane is decreased.

The mechanisms by which insulin induces the related transcription factor SREBP-1c are better understood. Insulin increases SREBP-1c transcription, processing, stability, and activity (33, 41); consequently, SREBP-1c, and its targets are decreased in LIRKO and streptozotocin-treated mice. However, SREBP-1c, its lipogenic targets, lipogenesis, and triglyceride content are markedly increased in the livers of *ob/ob* mice, despite the fact that they are severely insulin resistant. This has led to the hypothesis that obesity/type 2 diabetes is a state of selective insulin resistance, in which insulin resistance does not uniformly affect all branches of insulin signaling (42). Thus, some actions of insulin, such as the induction of lipogenesis, are largely preserved and driven to excess by the hyperinsulinemia present, whereas others, such as the suppression of gluconeogenesis, are markedly impaired. We would suggest that the effects of insulin on SREBP-2 and the cholesterologenic genes are affected to an intermediate extent; they do not become resistant to insulin, like the gluconeogenic genes, but they also do not remain as sensitive to insulin as SREBP-1c and the lipogenic genes. This would account for the relatively normal levels of SREBP-2 and cholesterologenic gene expression in *ob/ob* livers (43, 44).

Cholesterol synthesis is also subject to posttranscriptional control (45). Thus, despite relatively normal levels of SREBP-2 and mRNA levels of the cholesterologenic enzymes, cholesterol synthesis tends to be decreased in the livers of *ob/ob* mice and other rodent models of type 2 diabetes (46). However, in humans, obesity and type 2 diabetes appear to be associated with increased cholesterol synthesis (47).

Our finding that insulin deficiency strongly perturbs the statin/SREBP-2 axis could be relevant to patients with type 1 diabetes. Both rodents (48–50) and patients (51, 52) with type 1 diabetes show a decrease in cholesterol synthesis. To the extent that this is due to a defect in hepatic cholesterol synthesis, we would expect that the response to statins would be compromised. This matter is particularly timely given recent recommendations that all patients with type 1 diabetes be prescribed statins (53).

In summary, our data show that insulin regulates SREBP-2 and cholesterol synthesis, and is necessary for the changes in cholesterol synthesis observed with fasting and feeding, as well as the response to statin monotherapy. Recently, insulin has been shown to regulate SREBP-2 in tissues other than the liver, such as the brain (54). Moreover,

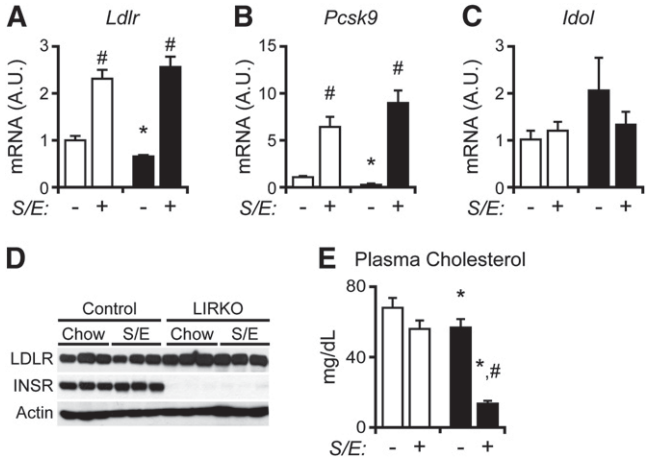

Fig. 6. Statin/ezetimibe treatment reduces plasma cholesterol in LIRKO mice. LIRKO and control mice were fed a diet with or without supplementation with statin/ezetimibe (S/E) (0.1% lovastatin and 0.025% ezetimibe) for 1 week and were euthanized in the nonfasted state. A–C: Gene expression was measured using real-time PCR. D: LDLR protein levels were measured in liver homogenates. E: Cholesterol was measured in the plasma of mice fasted for 4 h after 5 days on the diet with or without statin/ezetimibe supplementation. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same treatment conditions; $^{\#}P < 0.05$ versus untreated mice of the same genotype.)

SREBP-2 has been shown to be important for processes other than cholesterol synthesis, such as autophagy (55). Thus, SREBP-2 may prove to be a general mediator of insulin action throughout the body.

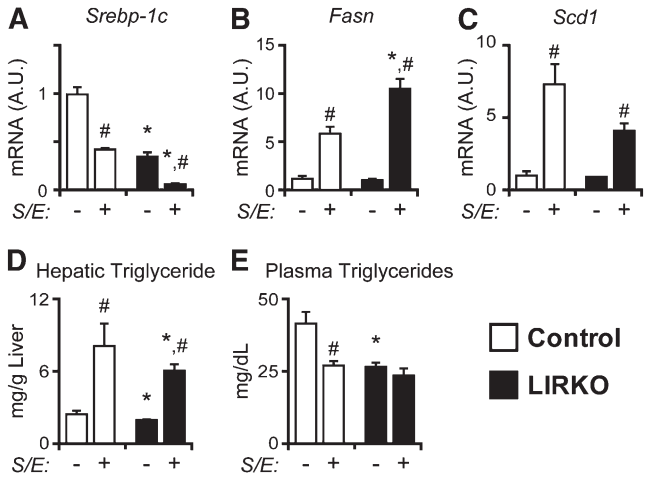

Fig. 7. Statin/ezetimibe treatment alters lipogenic gene expression in control and LIRKO livers. LIRKO and control mice were fed a diet with or without supplementation with statin/ezetimibe (S/E) (0.1% lovastatin and 0.025% ezetimibe) for 1 week and were euthanized in the nonfasted state. A–C: Gene expression was measured using real-time PCR. D: Hepatic triglyceride levels were measured at the time of euthanasia. E: Plasma triglycerides were measured in mice fasted for 4 h after 5 days on the diet with or without statin/ezetimibe supplementation. (n = 4–8; $^{*}P < 0.05$ versus control mice under the same feeding conditions; $^{\#}P < 0.05$ versus untreated mice of the same genotype.)

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