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Loss of von Hippel-Lindau Protein (VHL) Increases Systemic Cholesterol Levels through Targeting Hypoxia-Inducible Factor 2α and Regulation of Bile Acid Homeostasis

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Cholesterol synthesis is a highly oxygen-dependent process. Paradoxically, hypoxia is correlated with an increase in cellular and systemic cholesterol levels and risk of cardiovascular diseases. The mechanism for the increase in cholesterol during hypoxia is unclear. Hypoxia signaling is mediated through hypoxia-inducible factor 1α (HIF-1α) and HIF-2α. The present study demonstrates that activation of HIF signaling in the liver increases hepatic and systemic cholesterol levels due to a decrease in the expression of cholesterol hydroxylase CYP7A1 and other enzymes involved in bile acid synthesis. Specifically, activation of hepatic HIF-2α (but not HIF-1α) led to hypercholesterolemia. HIF-2α repressed the circadian expression of Rev-erbα, resulting in increased expression of E4BP4, a negative regulator of *Cyp7a1*. To understand if HIF-mediated decrease in bile acid synthesis is a physiologically relevant pathway by which hypoxia maintains or increases systemic cholesterol levels, two hypoxic mouse models were assessed, an acute lung injury model and mice exposed to 10% O_2 for 3 weeks. In both models, cholesterol levels increased with a concomitant decrease in expression of genes involved in bile acid synthesis. The present study demonstrates that hypoxic activation of hepatic HIF-2α leads to an adaptive increase in cholesterol levels through inhibition of bile acid synthesis.

Cholesterol is an essential nutrient important in many metabolic processes, and its levels are tightly regulated. Cholesterol synthesis is a highly oxygen-dependent process. The final step from lanosterol to cholesterol conversion requires nine molecules of dioxygen (1). However, systemic hypoxia is associated with an increase in cholesterol levels. Individuals living at high altitude are at high risk for coronary heart disease due to elevated low-density lipoprotein and total cholesterol levels caused by high-altitude hypoxia (2). In chronic hypoxic diseases, such as in patients with obstructive sleep apnea, no change or an increase in serum and liver cholesterol levels are observed, which can be reversed by continuous positive airway pressure (3–5). Moreover, mice exposed to chronic intermittent hypoxia (CIH) have a significant increase in total cholesterol levels (6, 7). These studies suggest that hypoxia-signaling pathways activate an adaptive response important in cholesterol homeostasis. Several mechanisms were proposed to account for hypoxia-induced dyslipidemia (8, 9). However, the mechanisms by which hypoxia regulates cholesterol homeostasis are not clear. Hypoxic signaling is mediated by an oxygen-sensitive transcription factor, hypoxia-inducible factor (HIF), consisting of two isoforms, HIF-1α and HIF-2α (10). Activation of HIF-1α induces glycolytic genes, whereas activation of HIF-2α regulates the expression of genes that are involved in fatty acid synthesis, fatty acid uptake, inflammation, fibrosis, and vascular tumors (9, 11–14).

In this study, liver hypoxia-induced dyslipidemia was shown to be associated with spontaneous hypercholesterolemia. Consistent with previous reports, the increase in hepatic cholesterol by hypoxia was not due to alterations in cholesterol synthesis, intestinal absorptive genes, or mobilization from fat stores (15, 16). Disruption of *Vhl* led to a HIF-2α-dependent decrease in cholesterol hydroxylases involved in the conversion of cholesterol to bile acids. To confirm that the decrease in cholesterol hydroxylase is an important mechanism to maintain cholesterol levels during hypoxia, two systemic hypoxic mouse models were used: mice exposed to normobaric 10% O_2 for 3 weeks and a mouse model of lung contusion (17). Both models exhibited significantly elevated levels of serum cholesterol with a parallel decrease in expression of genes involved in bile acid synthesis. Taken together, this study demonstrates a mechanistic basis for the adaptive maintenance of cholesterol levels during hypoxia. Thus, inhibition of HIF-2α may be a relevant and novel therapeutic target to ameliorate hypercholesterolemia during hypoxia.

MATERIALS AND METHODS

Animals and diets. $Vhl^{F/F}$, Vhl^{LivKO}, $Vhl^{\Delta Int}$, $Vhl/Arnt^{LivKO}$, $Vhl/Hif1\alpha^{LivKO}$, $Vhl/Hif2\alpha^{LivKO}$, $Vhl^{\Delta M\phi}$, and ODD-luc mice were described previously (11, 18–22). $Vhl^{\Delta M\phi}$ mice were generated by crossing LysM cre mice with $Vhl^{F/F}$ mice. All mice were fed *ad libitum* and kept in a 12-h dark/light cycle. Mice were fed with either a regular chow diet or an atherogenic (35 kcal% fat, 1% cholesterol, 0.5% cholic acid; Research Diets, New Brunswick, NJ), high-fat (~45 kcal% derived from fat; Research Diets), or ethanol (6% Lieber-DeCarli diet; Bio-Serv, Frenchtown, NJ) diet. For systemic hypoxia experiments, C57BL/6 mice in standard cages were placed in a normobaric hypoxia cham-

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ber with 10% O_2 for 3 weeks. Lung contusion (C57BL/6 mice) and *in vivo* imaging (ODD-luc mice) were performed as described previously (17, 23). All mice were sacrificed at 7:00 p.m. unless otherwise mentioned in the figure legends. All animal studies were carried out in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Western blot analysis. Microsomes and whole-cell extracts were prepared as previously described (21). Proteins were separated by SDS-PAGE. Microsomes were used for detection of CYP7A1 (Abcam, Cambridge, MA) and normalized to total protein by Coomassie blue staining.

Primary hepatocyte isolation. For primary hepatocyte isolation, livers were perfused continuously with 10 ml of Earle's balanced salt solution (EBSS) containing 0.5 mM EGTA, followed by serum-free L-15 medium containing collagenase type II (Worthington, Invitrogen). Hepatocytes were then harvested by gentle teasing and filtered through a 100-μm filter. Viable hepatocytes were plated in 6-well plates at a cell density of 4×10^5 in Williams E medium containing 10% fetal bovine serum (FBS). Five hours after plating, dead cells were removed and media refreshed. For adenovirus treatment, the desired amounts of Ad-Cyp7a1, Ad-HNF4α, and Ad-LRH-1 virus were added after 5 h of plating and then incubated until further analysis.

Cholesterol assay. For cholesterol extraction, 50 mg of liver or white adipose tissue was homogenized in 1 ml of chloroform-methanol (2:1). Primary hepatocytes were collected in 300 μl methanol, and then 600 μl of chloroform was added and vortexed. The homogenates were then centrifuged, and 200 μl of water was added to the supernatant and vortexed. The organic phase separated by centrifugation was dried overnight and resuspended in isopropanol. Cholesterol measurements were performed using the Cholesterol E kit (Wako Diagnostics, VA). Serum cholesterol was measured in 20 μl of serum, and lipoprotein profiles were assessed by fast performance liquid chromatography as previously described (24).

Bile acid assay. Bile acids from liver were extracted as described by Cheng et al. (25). Specific bile salt species were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) performed on a PESCIEX API200 ESI triple-quadrupole mass spectrometer (PerkinElmer Life Sciences) as described previously (26).

RNA extraction and qPCR analysis. RNA extraction and gene expression analysis using quantitative real-time PCR (qPCR) were performed as previously described (9). Primers are included in Table S1 in the supplemental material.

Statistical analysis. Results are expressed as means ± standard deviations (SD). *P* values were calculated by independent *t* test, 1-way analysis of variance (ANOVA), or 2-way ANOVA.

RESULTS

Disruption of hepatic VHL results in hypercholesterolemia. In order to elucidate the role of HIFs in cholesterol metabolism, serum cholesterol levels were assessed in mice with a conditional disruption of VHL in tissues or cells important in cholesterol homeostasis. Under normoxia, the von Hippel-Lindau tumor suppressor protein binds to hydroxylated HIFs and targets them for ubiquitination and degradation (27, 28). Genetic disruption of *Vhl* increases the expression of HIFs and HIF target genes (11). Disruption of VHL in the macrophage ($Vhl^{\Delta M\Phi}$) or the intestine ($Vhl^{\Delta Int}$) did not affect serum cholesterol levels compared to those of wild-type littermates ($Vhl^{F/F}$) (Fig. 1A). However, a temporal disruption of VHL in hepatocytes (Vhl^{LivKO}) using a tamoxifen-inducible Cre recombinase (9) significantly elevated serum cholesterol (Fig. 1A), hepatic cholesterol (Fig. 1B), and cholesterol content in isolated primary hepatocytes (Fig. 1C) compared to tamoxifen-treated wild-type littermates ($Vhl^{F/F}$). Hypercholesterolemia is primarily associated with increases in very low-density lipoprotein (VLDL) and LDL cholesterol, while high-density lipoprotein (HDL) cholesterol was decreased in Vhl^{LivKO} mice (Fig. 1D). Vhl^{LivKO} mice on a normal chow diet die within 30 days after *Vhl* disruption (Fig. 1E). Feeding an atherogenic diet markedly decreased the survival of Vhl^{LivKO} mice, which was not observed in Vhl^{LivKO} mice fed a high-fat or ethanol diet (Fig. 1F). We have previously reported that disruption of *Vhl* in the liver results in progressive liver steatosis and fibrosis (9). Therefore, it is possible that the hypercholesterolemia augments the progressive liver disease in Vhl^{LivKO} mice. In order to investigate the effect of atherogenic diet on serum cholesterol, Vhl^{LivKO} mice were injected with tamoxifen to disrupt *Vhl* expression and then immediately placed on the atherogenic diet for 1 week. The atherogenic diet further augmented the serum cholesterol levels in Vhl^{LivKO} mice (Fig. 1F). Together, these data demonstrate that disruption of *Vhl* in the liver leads to dysregulated cholesterol homeostasis.

Genes involved in *de novo* cholesterol synthesis in the liver and cholesterol absorption in the intestine are not altered by hepatic *Vhl* disruption. Hepatic cholesterol homeostasis is maintained by coordinated regulation of uptake, synthesis, and oxidative metabolism to bile acids (29). In order to determine the mechanism of hypercholesterolemia in Vhl^{LivKO} mice, expression of key enzymes involved in cholesterol synthesis and esterification were analyzed. Expression of *Cyp51*, HMG-coenzyme A (CoA) synthase (*Hmgcs*), HMG-CoA reductase (*Hmgr*), *Scd1*, and *Scd2* were significantly repressed 2 weeks after *Vhl* disruption but not at 5 days (Fig. 2A). The expression of sterol regulatory element binding protein 2 (*Srebp2*), a transcription factor critical in cholesterol synthesis, was significantly decreased at both 5 days and 2 weeks following *Vhl* disruption (Fig. 2A). These data demonstrate that the cholesterol-mediated negative feedback repression on *de novo* synthesis is still intact (30), and cholesterol synthesis is not the major mechanism by which liver disruption of VHL induces hypercholesterolemia. Cholesterol is cleared by the liver via the LDL receptor, and loss of function or genetic ablation of the LDL receptor results in hypercholesterolemia (31). Therefore, the dynamic changes in hepatic cholesterol levels and LDL receptor expression were assessed in Vhl^{LivKO} mice. A progressive and significant increase in hepatic cholesterol levels was observed starting at 3 days following *Vhl* disruption (Fig. 2B); however, LDL receptor protein levels decreased significantly only 2 weeks after *Vhl* disruption (Fig. 2C). Loss of LDL receptor after 2 weeks could be attributed to the negative-feedback repression exerted by increased intrahepatic cholesterol levels to limit cholesterol overload (32, 33). In addition, disruption of *Vhl* in liver did not affect the expression of *Npc1l1* (Fig. 2D), a gene involved in intestinal cholesterol absorption (34). Increasing cholesterol storage by adipose tissue improves the lipid profile in mice; on the other hand, cholesterol mobilization from adipose tissue is associated with hypercholesterolemia in humans (35, 36). The lack of a significant difference in white adipose cholesterol content between Vhl^{LivKO} and $Vhl^{F/F}$ mice suggests that hepatic VHL disruption does not affect adipose tissue cholesterol homeostasis (Fig. 2E).

CYP7A1 expression is repressed by hepatic *Vhl* disruption. Cholesterol is cleared by the liver through conversion to bile acids (37). CYP7A1 is the major rate-limiting cholesterol hydroxylase involved in the classical pathway of bile acid synthesis. In order to assess whether hypercholesterolemia is due to altered bile acid homeostasis, CYP7A1 expression was assessed in Vhl^{LivKO} mice. Two weeks after disruption of *Vhl*, expression of *Cyp7a1* mRNA was significantly reduced in the livers of Vhl^{LivKO} mice (Fig. 3A). Similarly, CYP7A1 mRNA and protein levels were significantly de-

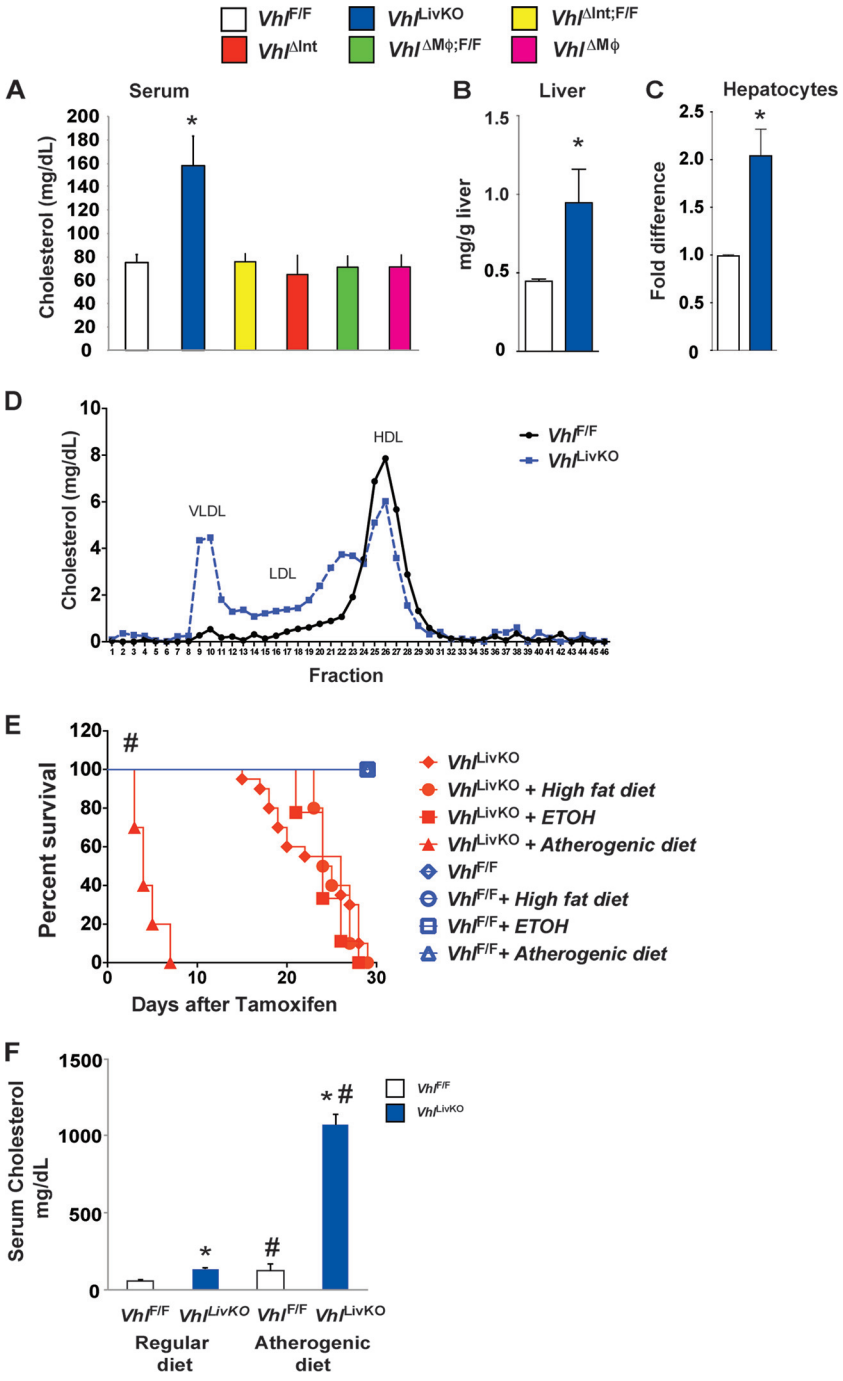

FIG 1 Disruption of hepatic VHL in the liver results in hypercholesterolemia and decreased survival. (A) Serum cholesterol levels in $Vhl^{F/F}$, Vhl^{LivKO}, $Vhl^{\Delta M\Phi}$, and $Vhl^{\Delta Int}$ mice 2 weeks after tamoxifen treatment. Cholesterol content was measured in the liver (B) and primary hepatocytes (C) from $Vhl^{F/F}$ and Vhl^{LivKO} mice. (D) Lipoprotein profile was assessed in the serum from $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. (E) Survival curve of $Vhl^{F/F}$ and Vhl^{LivKO} mice fed with regular chow, high-fat, ethanol (ETOH), or atherogenic diet. (F) Serum cholesterol levels measured in $Vhl^{F/F}$ and Vhl^{LivKO} mice on chow or atherogenic diet for 1 week. Five to seven mice were assessed per treatment group. Each bar graph represents the mean values ± SD. **, $P < 0.01$ versus $Vhl^{F/F}$; #, $P < 0.01$ versus regular diet.

creased in primary hepatocytes from Vhl^{LivKO} mice (Fig. 3A and B). To identify mechanisms by which disruption of *Vhl* repress CYP7A1 expression, several known pathways important in the regulation of CYP7A1 were assessed. Activation of JNK induces hypercholesterolemia and cholestasis (38) and is a potent inhibitor of *Cyp7a1* expression (39). Consistent with inflammation in the livers of Vhl^{LivKO} mice (9), JNK phosphorylation was significantly increased at 5 days and 2 weeks after disruption of *Vhl* (Fig. 3C). However, JNK inhibitor (SP 600125) did not rescue *Cyp7a1* mRNA levels in primary hepatocytes from Vhl^{LivKO} mice (Fig. 3D).

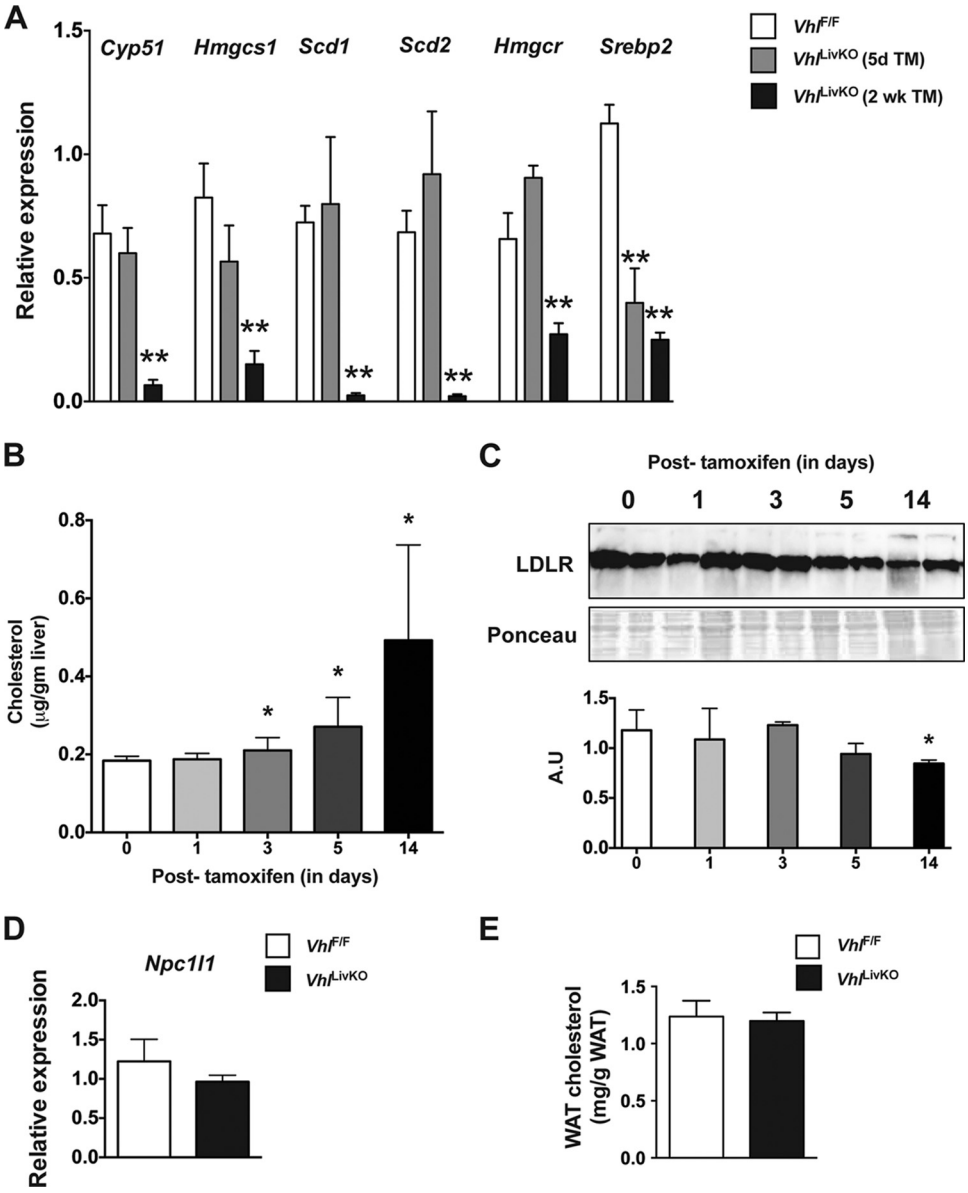

FIG 2 Cholesterol synthesis, absorption, and mobilization are not affected by the hepatic disruption of VHL. (A) qPCR analysis of cholesterol synthesis genes in $Vhl^{F/F}$ and Vhl^{LivKO} livers 5 days or 2 weeks following tamoxifen treatment. Hepatic cholesterol (B) and LDL receptor expression (C) were analyzed and quantitated (bottom inset) in the livers of Vhl^{LivKO} mice. (D) qPCR analysis of *Npc1l1* in the small intestine of $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. mRNA was normalized to β-*actin*. (E) White adipose tissue cholesterol from $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. Five to nine mice were assessed per treatment group. Each bar graph represents the mean values ± SD. **, $P < 0.01$ compared to $Vhl^{F/F}$ mice.

Similarly, adenovirus-mediated overexpression of HNF-4α or LRH-1, known CYP7A1 transcriptional regulators (40), did not rescue the expression of *Cyp7a1* mRNA levels in primary hepatocytes from Vhl^{LivKO} mice (Fig. 3E).

Hepatic VHL disruption alters circadian oscillation of CYP7A1. Cholesterol exhibits diurnal variation due to circadian oscillation in the expression of genes involved in cholesterol metabolism, including CYP7A1 (41). In order to investigate whether CYP7A1 expression and circadian regulation was altered by HIF activation, Vhl^{LivKO} and littermate control mice were treated with tamoxifen for 5 days and 2 weeks and killed at 7:00 a.m. and 7:00 p.m. CYP7A1 mRNA and protein levels were not changed at 7:00 a.m.; however, the circadian increase at 7:00 p.m. was significantly repressed in the Vhl^{LivKO} mice at 5 days (Fig. 4A). However, circadian expression of other cholesterol hydroxylases, such as *Cyp27a1*, *Cyp7b1*, *Hsd3b1*, and *Cyp3a11*, was not affected by disruption of *Vhl* (Fig. 4B). Furthermore, 2 weeks following VHL disruption, CYP7A1 mRNA and protein at 7:00 a.m. and the circadian induction at 7:00 p.m. were significantly repressed (Fig. 4C). Among the various key transcription factors that regulate circadian genes, Rev-erbα plays an essential role in the diurnal expression of *Cyp7a1* (42, 43). The circadian induction of Rev-erbα is inhibited in the Vhl^{LivKO} mice at both 5 days and 2 weeks following tamoxifen treatment (Fig. 4C). Consistent with the protein expression, the circadian induction of *Rev-erbα* but not *Rev-erbβ* was significantly repressed at 5 days after disruption of *Vhl*

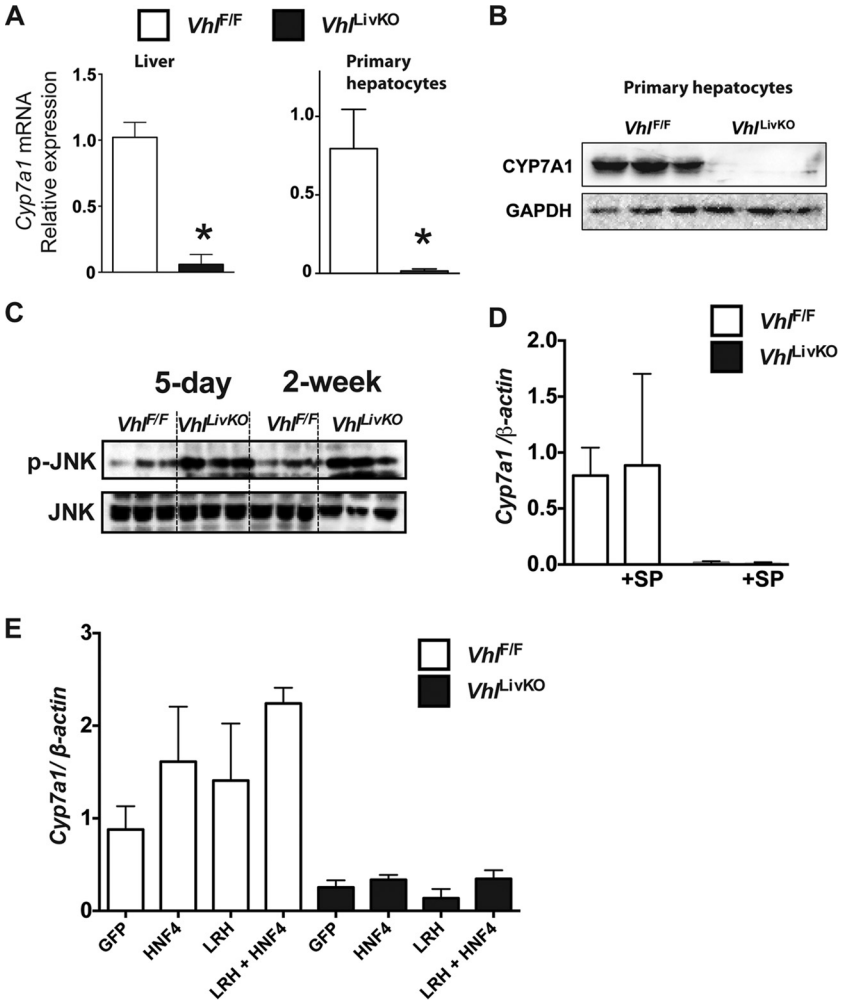

FIG 3 Loss of VHL in liver results in repression of CYP7A1 expression. (A) qPCR analysis examining the expression of *Cyp7a1* mRNA levels in the livers and primary hepatocytes of Vhl^{LivKO} mice. mRNA was normalized to β-*actin*. Four to six mice were assessed per treatment group. (B) Western analysis of CYP7A1 in primary hepatocytes from $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. (C) Western analysis examining the phosphorylation of JNK in the liver of Vhl^{LivKO} mice five days or 2 weeks after tamoxifen treatment. qPCR analysis examining the expression of *Cyp7a1* mRNA levels in the primary hepatocytes of Vhl^{LivKO} mice treated with the JNK inhibitor SP 600125 (SP) (D) or with recombinant adenovirus expressing HNF-4α or LRH-1 (E). mRNA was normalized to β-*actin*. *, $P < 0.05$ versus $Vhl^{F/F}$ mice.

(Fig. 4D). Moreover, the loss of expression of *Rev-erbα* in Vhl^{LivKO} mice at 7:00 p.m. resulted in a significant increase in *E4bp4* mRNA levels (Fig. 4D), a negative regulator of *Cyp7a1* (42). However, no difference in the expression of circadian genes such as *Bmal-1* and *Per-1* was observed by disruption of *Vhl* in liver (Fig. 4D). These data suggest that hypoxia-mediated circadian dysregulation leads to a decrease in CYP7A1 levels.

***Vhl* disruption in the liver leads to decreased expression of genes critical in bile acid synthesis.** To address whether repression of CYP7A1 is the primary mechanism for the aberrant cholesterol levels in Vhl^{LivKO} mice, CYP7A1 was overexpressed in primary hepatocytes from Vhl^{LivKO} mice. Rescuing CYP7A1 did not ameliorate cholesterol levels in the primary hepatocytes from Vhl^{LivKO} mice (Fig. 5A and B). Conversion of cholesterol to bile is a multienzymatic process involving numerous cholesterol hydroxylases. Further analysis revealed that disruption of VHL in livers resulted in repression of genes encoding other key cholesterol hydroxylases, such as *Cyp27a1*, *Cyp7b1*, *Cyp8b1*, *Hsd3b*, *Amacr*, *Cyp3a11*, *Cyp29a1*, and *Slc25c1* (Fig. 5C). Taken together, these data suggest that activation of hypoxia signaling by disruption of VHL results in a global decrease in cholesterol hydroxylases involved in bile acid synthesis.

***Vhl* disruption in the liver leads to decreased bile acids.** Decreases in the expression and activity of cholesterol hydroxylases decrease bile acid synthesis, leading to hypercholesterolemia. Therefore, to understand if repression of cholesterol hydroxylases by hypoxia signaling could adversely affect bile acid synthesis, the hepatic bile acid pool was measured in Vhl^{LivKO} mice. The total liver bile acid pool (Fig. 6A) and gallbladder size were significantly reduced in Vhl^{LivKO} mice (Fig. 6B). In addition, major hepatic bile acid species were significantly decreased, including the recently identified endogenous farsenoid X receptor (FXR) antagonist, tauro-β-muricholic acid (Fig. 6C) (44, 45). Bile acids inhibit their own synthesis by a negative feedback mechanism via activation of FXR (46, 47). Consistent with the decrease in its endogenous ligand, FXR target genes, such as *Abcg5*, *Abcg8*, small heterodimer

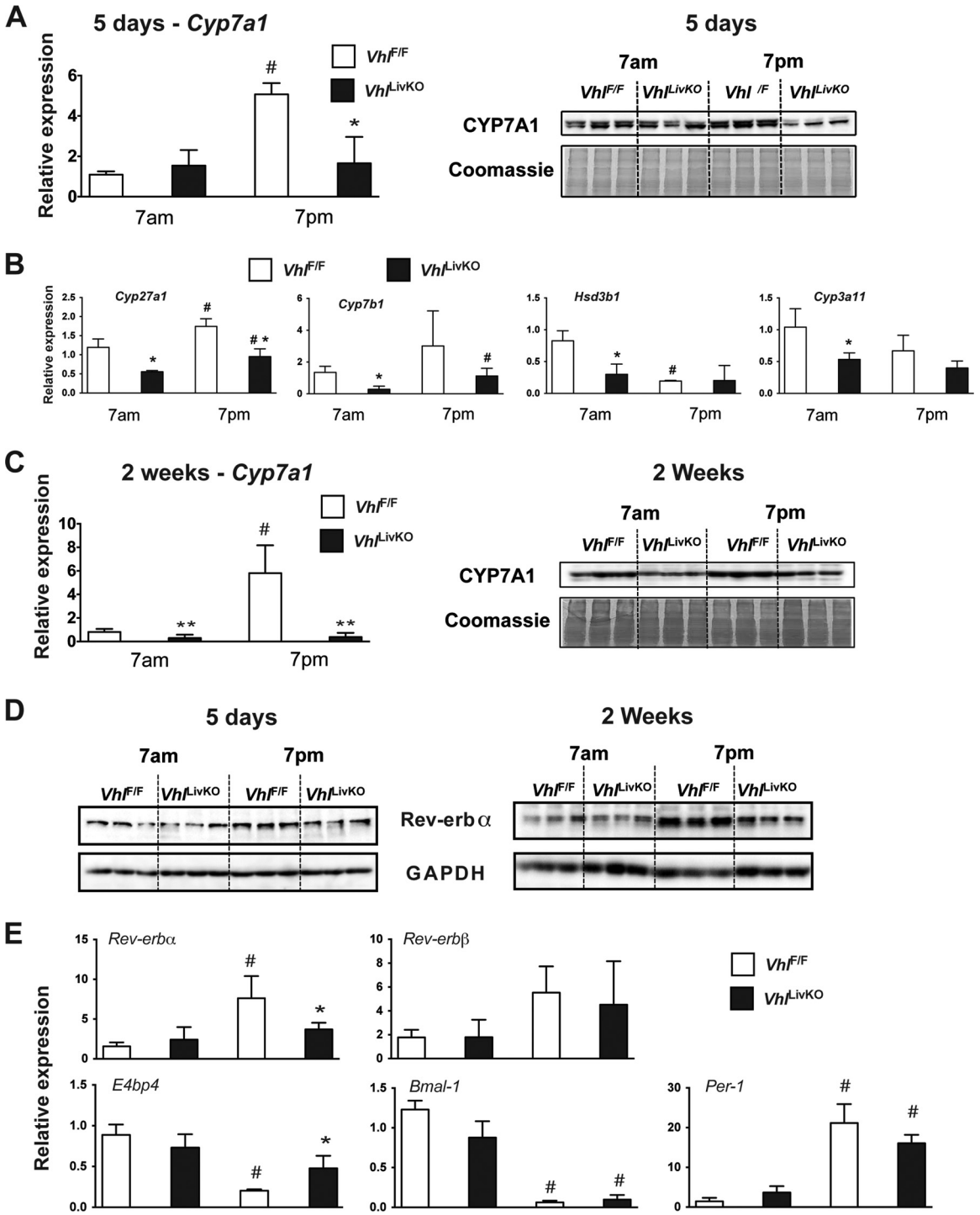

FIG 4 Loss of VHL disrupts circadian expression of CYP7A1. qPCR and Western blot analysis of CYP7A1 in the microsome fraction (A) and qPCR analysis of cholesterol hydroxylases (B) at 7:00 a.m. and 7:00 p.m. in $Vhl^{F/F}$ and Vhl^{LivKO} livers 5 days after *Vhl* disruption. (C) qPCR and Western blot analysis of CYP7A1 at 7:00 a.m. and 7:00 p.m. in $Vhl^{F/F}$ and Vhl^{LivKO} livers 2 weeks after *Vhl* disruption. mRNA was normalized to β-*actin*, and the microsome fraction was normalized by Coomassie blue staining. Four to six mice were assessed per treatment group. (D) Western blot analysis examining the circadian expression of Rev-erbα in $Vhl^{F/F}$ and Vhl^{LivKO} livers 5 days or 2 weeks after *Vhl* disruption. (E) qPCR analysis of circadian genes in $Vhl^{F/F}$ and Vhl^{LivKO} livers 5 days after *Vhl* disruption. mRNA was normalized to β-*actin*. Each bar graph represents the mean values ± SD. *, $P < 0.05$ versus $Vhl^{F/F}$ mice; **, $P < 0.01$ versus $Vhl^{F/F}$ mice; #, $P < 0.01$ versus results at 7:00 a.m.

partner (*Shp*), and bile salt export protein (*Bsep*) in the liver and *Fgf15* in the intestines, were significantly repressed in Vhl^{LivKO} mice (Fig. 6D) without changes in FXR protein levels (Fig. 6E). In addition, the expression of bile acid-CoA:amino acid *N*-acyltransferase (*Baat*) and the bile acid transporter sodium taurocholate cotransport protein (*Ntcp*) are significantly reduced in the livers of Vhl^{LivKO} mice (Fig. 6D). These data demonstrate that HIF activation disrupts bile acid synthesis and signaling.

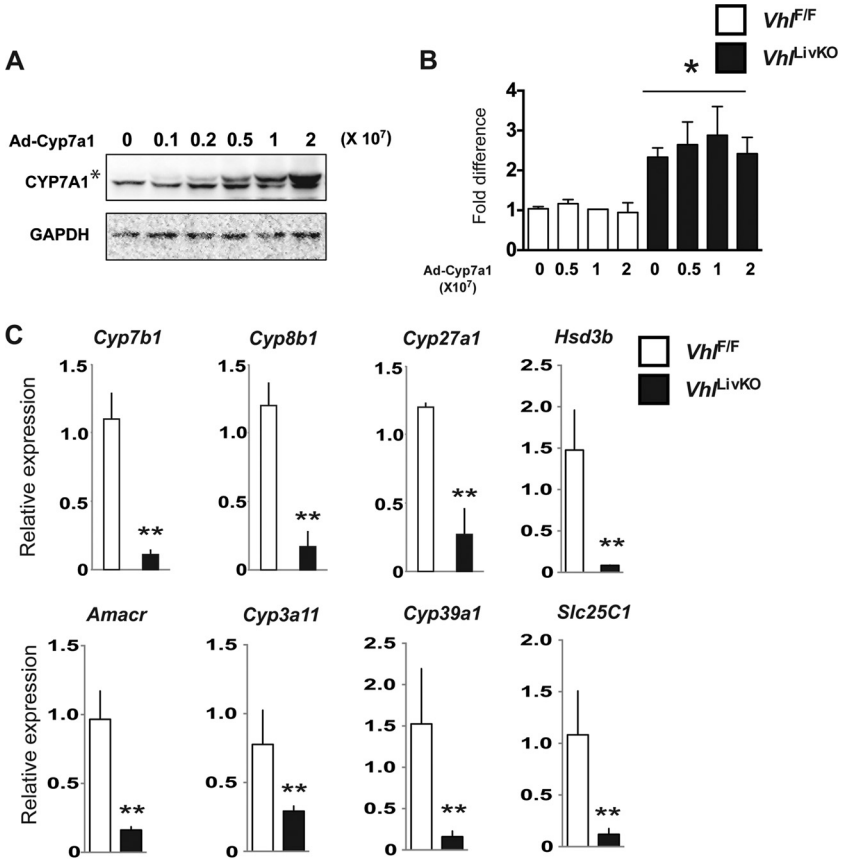

FIG 5 Adenoviral rescue of CYP7A1 did not ameliorate cholesterol levels in primary hepatocytes. Western blot analysis of CYP7A1 (A) and cholesterol assay in primary hepatocytes (B) treated with recombinant adenovirus expressing *Cyp7a1* in primary hepatocytes from $Vhl^{F/F}$ and Vhl^{LivKO} mice. (C) qPCR analysis of cholesterol hydroxylases in $Vhl^{F/F}$ and Vhl^{LivKO} livers 2 weeks after *Vhl* disruption. mRNA was normalized to β-*actin*. Each bar graph represents the mean values ± SD. *, $P < 0.05$ versus $Vhl^{F/F}$ mice; **, $P < 0.01$ versus $Vhl^{F/F}$ mice; #, $P < 0.01$ versus results at 7:00 a.m.

Repression of Cyp7a1 is HIF-2α dependent. Disruption of *Vhl* results in the overexpression of both HIF-1α and HIF-2α (11). HIF-1α and HIF-2α exert distinct functions by regulating different subsets of genes (48). Elevation in the serum and hepatic cholesterol observed in Vhl^{LivKO} was completely abolished in mice with compound disruption of VHL and HIF-2α (*Vhl/Hif-2α*^{LivKO}) and VHL and ARNT (*Vhl/Arnt*^{LivKO}) but not in mice with disruption of VHL and HIF-1α (*Vhl/Hif-1α*^{LivKO}), demonstrating that perturbation in cholesterol levels was HIF-2α dependent (Fig. 7A and B). Concordant with the improvement in cholesterol homeostasis, CYP7A1 and Rev-erbα expression were rescued by combined disruption of VHL and HIF-2α (Fig. 7C). In addition, combined disruption of VHL and HIF-2α restored the expression of bile acid synthesis and FXR target genes (Fig. 7D and E). These data demonstrate that circadian regulation of cholesterol hydroxylases by HIF-2α plays a key role in cholesterol and bile homeostasis following hypoxia.

Systemic hypoxia leads to hypercholesterolemia and a decrease in bile synthesis. To further demonstrate that liver hypoxia signaling is a critical regulator of cholesterol homeostasis, a model of closed-chest unilateral lung contusion that rapidly induces systemic hypoxia was analyzed (49). Lung contusion induced a robust increase in systemic hypoxia 24 h following injury, as assessed in a hypoxia reporter mouse model, ODD-luc mice (Fig. 8A) (18, 23). In parallel with the systemic hypoxia, serum and liver cholesterol levels were significantly elevated at 24 and 48 h after the insult (Fig. 8B). However, the decrease in hepatic bile acid levels did not reach significance, raising the possibility that enterohepatic recycling of bile compensates for the short-term inhibition of bile acid synthesis (Fig. 8B). Systemic hypoxia decreased the levels of CYP7A1 protein in the microsomal fraction of liver 24 h after the initiation of lung contusion (Fig. 8C). In addition, mRNA levels of *Cyp7a1*, *Cyp7b1*, and *Cyp27a1* were significantly decreased in livers at 24 and 48 h after lung injury (Fig. 8D). Further analysis revealed that the circadian gene *Rev-erbα* is significantly repressed at 48 h after lung injury with a concomitant increase in *E4bp4* expression (Fig. 8D). To investigate whether chronic systemic hypoxia can affect cholesterol and bile acid homeostasis, C57BL/6 mice were exposed to 10% O_2 for 3 weeks. Hypoxia significantly increased the serum and liver cholesterol levels and decreased hepatic bile acids (Fig. 8E). Moreover, the expression of cholesterol hydroxylases, such as *Cyp7a1*, *Cyp8b1*, and *Cyp27a1*, was significantly repressed (Fig. 8F). Similar to the lung contusion model, chronic hypoxia for 3 weeks significantly decreased *Rev-erbα* expression, resulting in increased expression of *E4bp4* (Fig. 8F). The data demonstrate that activation of hypoxia signaling in liver increases hepatic and systemic cholesterol levels. Moreover, the data suggest that the adaptive increase in systemic cholesterol levels following hypoxia are due to a decrease in cholesterol clearance as bile acids.

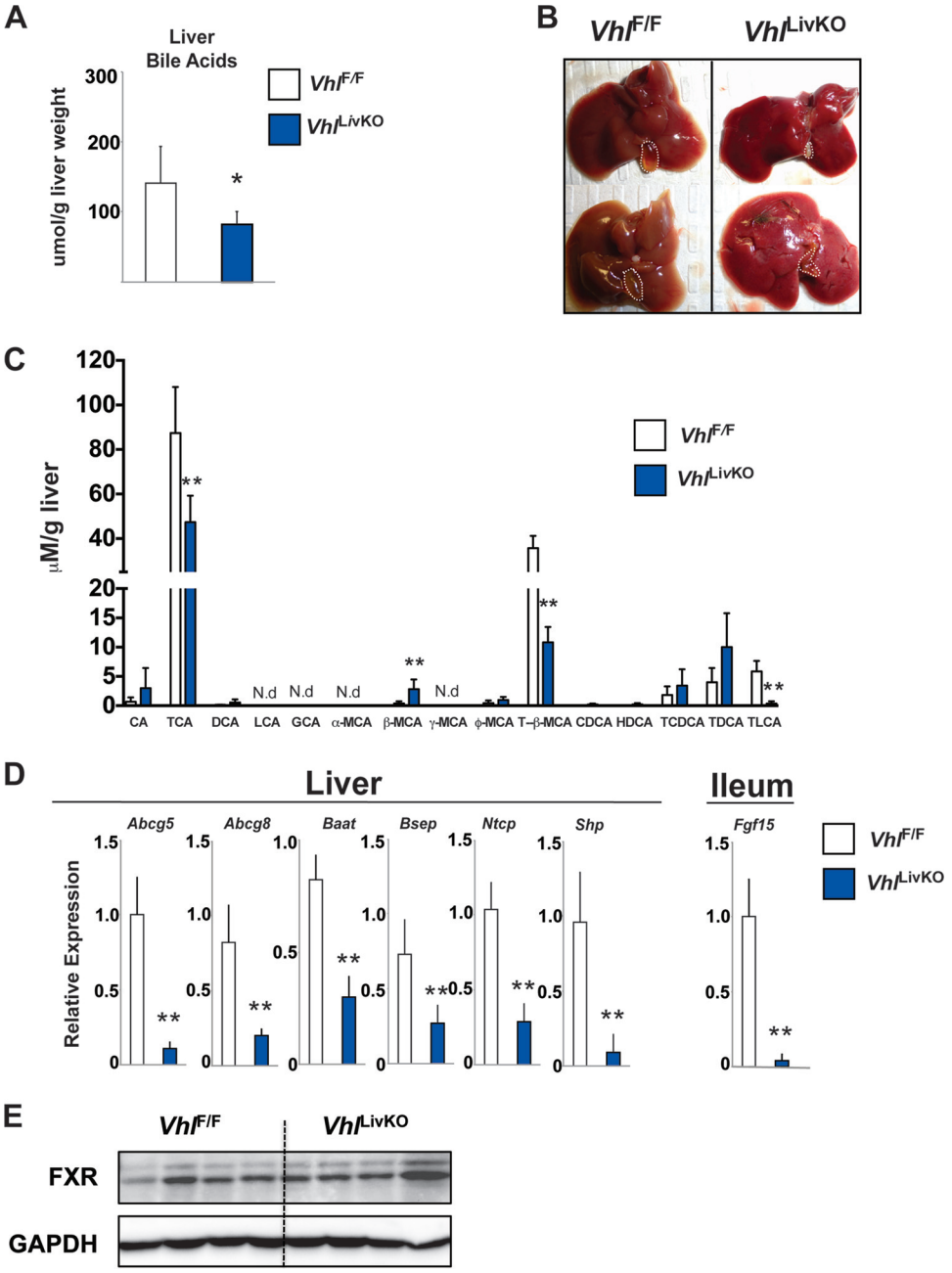

FIG 6 Disruption of liver VHL decreases bile acid and FXR signaling. (A) Hepatic bile acids in $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. (B) Gross morphology of the liver and gallbladder in $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. (C) Bile acid species quantitation in livers of $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. CA, cholic acid; TCA, taurocholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; GCA, glycocholic acid; MCA, muricholic acid; T-β-MCA, tauro-β-muricholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid. N.D, not detected. (D) FXR target genes were assessed by qPCR in the liver and ileum of $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks following tamoxifen treatment. Expression was normalized to β-*actin*. Five to eight mice were assessed per treatment group. Each bar graph represents the mean values ± SD. (E) Western blot analysis of FXR in livers of $Vhl^{F/F}$ and Vhl^{LivKO} mice 2 weeks after tamoxifen treatment. *, $P < 0.05$ versus $Vhl^{F/F}$ mice; **, $P < 0.01$ versus $Vhl^{F/F}$ mice.

DISCUSSION

Cholesterol synthesis is a multienzymatic reaction which requires energy and oxygen. It is hypothesized that the evolution of cholesterol is directly correlated to atmospheric O_2 (50). However, during hypoxia, adaptive mechanisms exist to maintain systemic cholesterol levels. Populations indigenous to high altitude, sleep apnea patients, and mouse models exposed to CIH have an increase in cholesterol levels and are at higher risk for cardiovascular diseases (2, 4, 5). It is worth noting that continuous positive airway pressure is the effective treatment for hypercholesterolemia in obstructive sleep apnea patients (3), further raising the possibility that hypoxia signaling is involved in the regulation of cholesterol

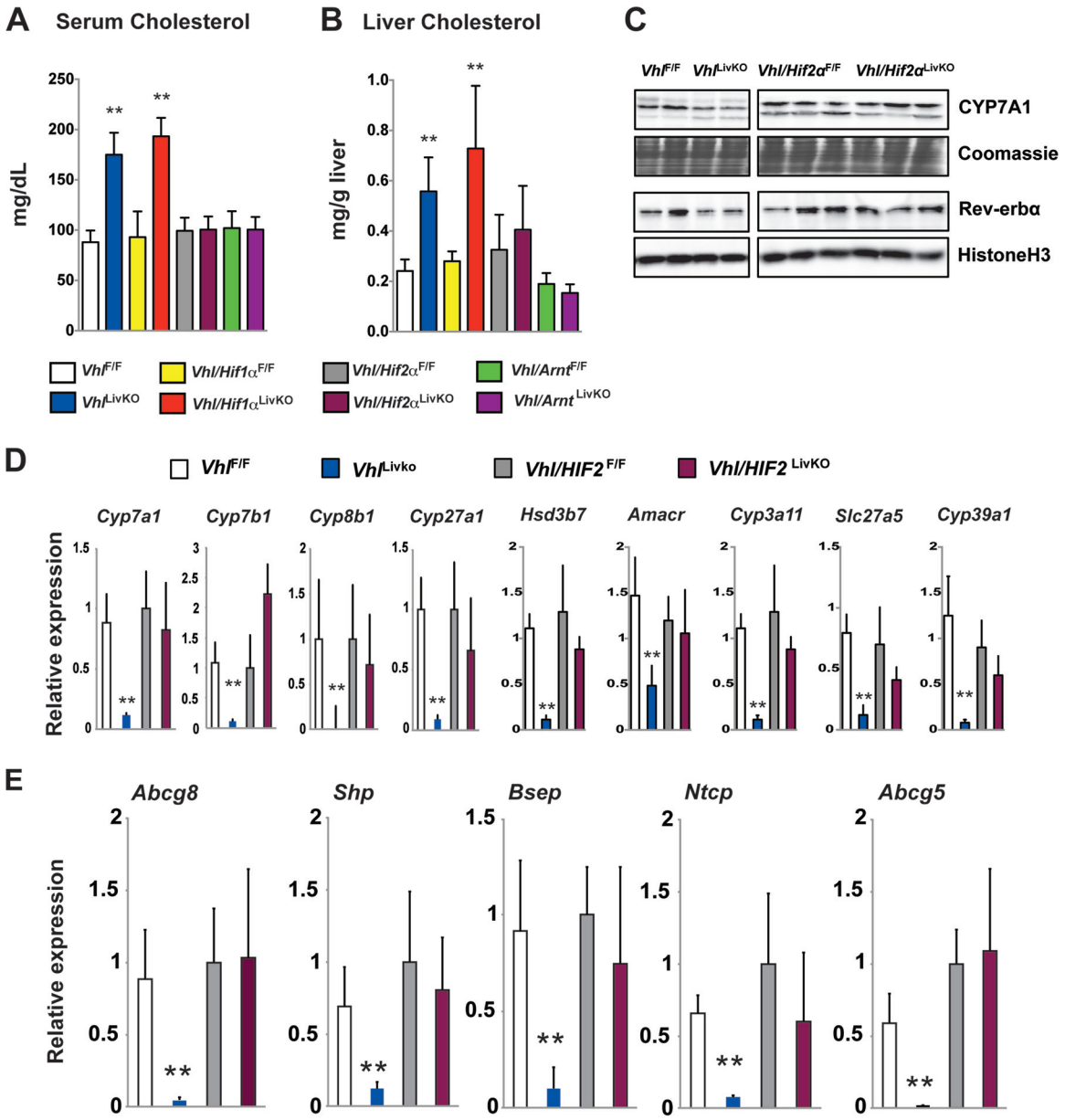

FIG 7 Liver HIF-2α induces hypercholesterolemia and inhibits bile acid synthesis. Serum (A) and liver cholesterol (B) analyzed in $Vhl^{F/F}$, Vhl^{LivKO}, $Vhl/Hif1\alpha^{F/F}$, $Vhl/Hif1\alpha^{LivKO}$, $Vhl/Hif2\alpha^{F/F}$, and $Vhl/Hif2\alpha^{LivKO}$ mice 2 weeks following tamoxifen treatment. (C) Western blot analysis examining the expression of CYP7A1 and Rev-erbα in the microsome fraction and nuclear lysates and qPCR analysis for bile acid synthesis genes (D) and FXR target genes (E) in livers of $Vhl^{F/F}$, Vhl^{LivKO}, $Vhl/Hif2\alpha^{F/F}$, and $Vhl/Hif2\alpha^{LivKO}$ mice 2 weeks after tamoxifen treatment. Expression was normalized to β-*actin*. Five to nine mice were assessed per treatment group. Each bar graph represents the mean values ± SD. **, $P < 0.01$ versus $Vhl^{F/F}$ mice.

homeostasis. The mechanism by which hypoxia sustains or induces systemic cholesterol levels is not well understood. Using liver-specific HIF-overexpressing (Vhl^{LivKO}) or systemic hypoxic mouse models, we demonstrate that hypoxia-mediated perturbation in cholesterol homeostasis is due to a decrease in the expression of cholesterol hydroxylases involved in bile synthesis. Moreover, the data suggest that HIF-2α plays a critical role in this mechanism by regulating circadian expression of hepatic Rev-erbα and *Cyp7a1*.

Hypercholesterolemia in Vhl^{LivKO} mice is not due to cholesterol biosynthesis or uptake. An increase in the intrahepatic cholesterol levels precedes the changes in LDL receptor levels, suggesting a defective cholesterol metabolism at the cellular level as the primary cause for aberrant cholesterol levels in Vhl^{LivKO} mice. However, the secondary decrease in the LDL receptor levels could further augment the increase in VLDL and LDL cholesterol, leading to hypercholesterolemia in Vhl^{LivKO} mice. CYP7A1, the key rate-limiting enzyme in bile acid synthesis, was inhibited by hypoxia and/or HIF-2α activation. Mice that overexpress CYP7A1 have increased bile acid pools and lower serum cholesterol (51, 52). On the other hand, $Cyp7a1^{-/-}$ mice have decreased bile acid synthesis. These studies are consistent

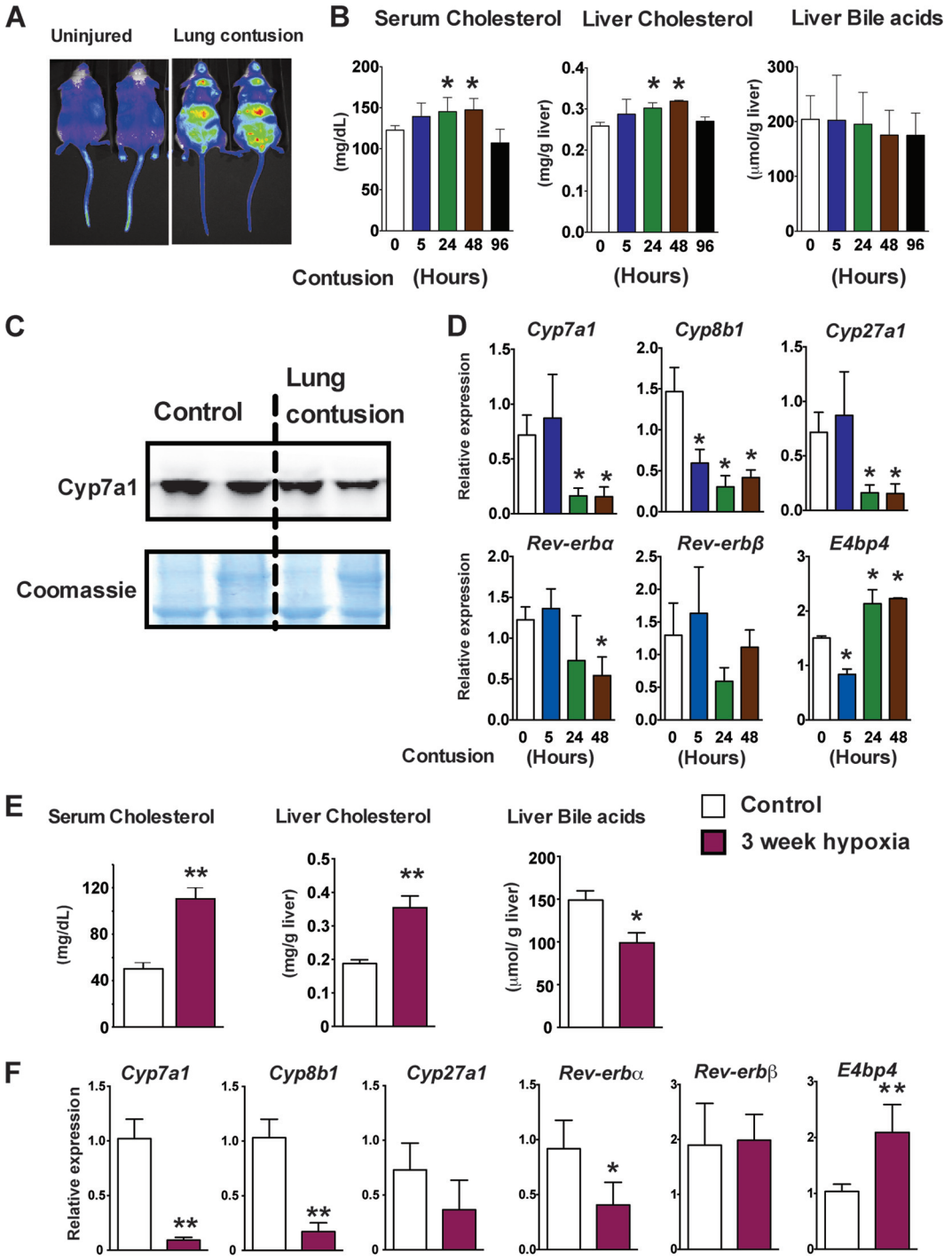

FIG 8 Chronic hypoxia and lung contusion increases cholesterol levels and decreases bile acid synthesis in mice. (A) *In vivo* imaging in Odd-luc mice demonstrating systemic hypoxia 24 h following lung contusion. (B) Serum cholesterol, liver cholesterol, and total liver bile acid measured at 5, 24, 48, and 96 h after induction of lung injury. (C) Western blot analysis of CYP7A1 in the microsome fractions 24 h after lung injury. Expression was normalized to Coomassie blue staining. (D) qPCR analysis in livers at 5, 24, and 48 h after lung injury. Expression was normalized to β-*actin*. Three to six mice were assessed per treatment group. (E) Serum cholesterol, liver cholesterol, and hepatic total bile acids measured in C57BL/6 mice exposed to 10% O_2 for 3 weeks. (F) qPCR analysis of bile acid synthesis and circadian genes in mice exposed to 10% O_2 for 3 weeks. Expression was normalized to β-*actin*. Four mice were assessed per treatment group. Each bar graph represents the mean values ± SD. *, $P < 0.05$ versus the control; **, $P < 0.01$ versus the control.

with the data presented in the manuscript. However, *Cyp7a1*$^{-/-}$ mice do not have any overt differences in hepatic cholesterol levels compared to wild-type mice (53). Similarly, adenovirus-mediated rescue of CYP7A1 alone did not ameliorate cholesterol levels in primary hepatocytes from *Vhl*^{LivKO} mice. Bile acid metabolism is profoundly repressed in isolated cultured mouse primary hepatocytes compared to the liver; therefore, we cannot exclude the possibility that CYP7A1 is the primary

hypoxia-repressed gene involved in hypercholesterolemia. However, a significant decrease in a battery of cholesterol hydroxylases in the liver of Vhl^{LivKO} mice suggests that global repression of other cholesterol hydroxylases, in addition to CYP7A1, is the essential mechanism that leads to dysregulated cholesterol levels following hypoxia.

Cholesterol levels exhibit circadian variation due to the diurnal expression of CYP7A1 (41). Rev-erbα is one of the key transcription factors that regulate the expression of various circadian genes. Rev-erbα knockout mice exhibit decreased bile acid levels due to loss of *Cyp7a1* expression (42, 43). The current study demonstrates that activation of HIF signaling disrupts the circadian expression of Rev-erbα, resulting in increased expression of *E4bp4*, a negative regulator of *Cyp7a1* (42). In addition, systemic hypoxia due to lung contusion or chronic hypoxia exposure of mice recapitulates the decrease in genes involved in bile acid synthesis and bile acid levels with a concomitant increase in systemic cholesterol levels. Furthermore, repression of CYP7A1 is also associated with loss of Rev-erbα and increase in *E4bp4* in these hypoxic mouse models (42). The expression of other cholesterol hydroxylases was not repressed by HIF-2α through disrupting circadian regulation. This suggests that disruption of VHL inhibits bile acid synthesis, perhaps through other parallel mechanisms that need to be further investigated. Together, these results suggest that perturbation in cholesterol homeostasis following hypoxia is an adaptive mechanism to increase systemic cholesterol levels in an HIF-2α-dependent manner. The profound effect of hypoxia signaling on cholesterol metabolism is through decreasing cholesterol clearance as bile acids via disrupting the circadian expression of CYP7A1 and by repressing downstream cholesterol hydroxylases through a currently unknown mechanism.

Atherosclerosis is a well-known inflammation and lipid-associated disease, and chronic intermittent hypoxia (CIH) exacerbates disease progression (6, 7, 54). An important mechanism that contributes to atherosclerosis is recruitment of macrophages that scavenge fatty acids, resulting in the formation of foam cells. Several studies have demonstrated the importance of HIF-1α in foam cell formation and atherosclerosis (55, 56). Moreover, recently it was shown that HIF-1α induction of *Angptl4* in adipose tissues contributes to the hypoxic potentiation of atherosclerosis (57). However, partial disruption of HIF-1α in adipose tissue did not improve hypercholesterolemia caused by CIH. The role of liver HIF-2α in hypoxia-mediated atherosclerosis has not yet been explored. CIH induces systemic inflammation, and recently we demonstrated that HIF-2α induces a proinflammatory response by increasing tumor necrosis factor alpha expression (58). The present work, together with our previous study (9), demonstrates that chronic hypoxic signaling through HIF-2α in the liver may initiate a proatherogenic environment by inducing inflammation and hypercholesterolemia.

The data in this study demonstrate that a decrease in cholesterol clearance as bile is the primary mechanism for the adaptive increase in liver and systemic cholesterol levels during hypoxia. Furthermore, HIF-2α regulation of the circadian gene *Rev-erbα* was demonstrated as a critical mechanism in cholesterol homeostasis. Since HIF-2α has an essential role in hepatic inflammation and liver cholesterol homeostasis, HIF-2α could be a therapeutic target to improve dyslipidemia and systemic inflammation in patients with increased risk of cardiovascular disease.

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We declare no conflicts of interest.

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