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BASIC AND TRANSLATIONAL—BILIARY

Activation of the Hypoxia Inducible Factor 1α Subunit Pathway in Steatotic Liver Contributes to Formation of Cholesterol Gallstones

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BACKGROUND & AIMS: Hypoxia-inducible factor 1α subunit (HIF1A) is a transcription factor that controls the cellular response to hypoxia and is activated in hepatocytes of patients with nonalcoholic fatty liver disease (NAFLD). NAFLD increases the risk for cholesterol gallstone disease by unclear mechanisms. We studied the relationship between HIF1A and gallstone formation associated with liver steatosis. **METHODS:** We performed studies with mice with inducible disruption of *Hif1a* in hepatocytes via a Cre adenoviral vector (inducible hepatocyte-selective HIF1A knockout [iH-HIFKO] mice), and mice without disruption of *Hif1a* (control mice). Mice were fed a diet rich in cholesterol and cholate for 1 or 2 weeks; gallbladders were collected and the number of gallstones was determined. Livers and biliary tissues were analyzed by histology, quantitative reverse-transcription polymerase chain reaction, immunohistochemistry, and immunoblots. We measured concentrations of bile acid, cholesterol, and phospholipid in bile and rates of bile flow. Primary hepatocytes and cholangiocytes were isolated and analyzed. HIF1A was knocked down in Hepa1-6 cells with small interfering RNAs. Liver biopsy samples from patients with NAFLD, with or without gallstones, were analyzed by quantitative reverse-transcription polymerase chain reaction. **RESULTS:** Control mice fed a diet rich in cholesterol and cholate developed liver steatosis with hypoxia; levels of HIF1A protein were increased in hepatocytes around central veins and 90% of mice developed cholesterol gallstones. Only 20% of the iH-HIFKO mice developed cholesterol gallstones. In iH-HIFKO mice, the biliary lipid concentration was reduced by 36%, compared with control mice, and bile flow was increased by 35%. We observed increased water secretion from hepatocytes into bile canaliculi to mediate these effects, resulting in suppression of cholelithogenesis. Hepatic expression of aquaporin 8 (AQP8) protein was 1.5-fold higher in iH-HIFKO mice than in control mice. Under hypoxic conditions, cultured hepatocytes increased expression of *Hif1a*, *Hmox1*, and *Vegfa* messenger RNAs (mRNAs), and down-regulated expression of AQP8 mRNA and protein; AQP8 down-regulation was not observed in cells with knockdown of HIF1A. iH-HIFKO mice had reduced inflammation and mucin deposition in the gallbladder compared with control mice. Liver tissues from patients with NAFLD with gallstones had increased levels of *HIF1A*, *HMOX1*, and *VEGFA* mRNAs, compared with livers from patients with NAFLD without gallstones. **CONCLUSIONS:** In steatotic livers of mice, hypoxia up-regulates expression of HIF1A, which reduces expression of AQP8 and concentrates biliary lipids via suppression of water secretion from hepatocytes. This promotes cholesterol gallstone formation. Livers from patients with NAFLD and gallstones express higher levels of HIF1A than livers from patients with NAFLD without gallstones.

Keywords: Gallstone; NAFLD; Hypoxia; Aquaporin-8.

Gallstone disease is very common and the annual treatment cost in the United States is approximately 6 billion dollars.[1] Furthermore, gallstone disease is a major risk factor for gallbladder cancer,[2] which has a very poor prognosis.[3] Therefore, reducing the prevalence of this

Abbreviations used in this paper: **AQP, aquaporin; CCD, cholesterol and cholate-rich diet; cDNA, complementary DNA; HIF1, hypoxia-inducible factor 1; HIF1α, hypoxia-inducible factor 1 α subunit; Hmox1, heme oxygenase-1; IBDU, isolated bile duct unit; iH-HIFKO, inducible hepatocyte-selective HIF1A knockout; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; TCA, taurocholate; Vegfa, vascular endothelial growth factor α.**

Most current article

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

NAFLD is a well-known risk factor of gallstones. The mechanistic links between NAFLD and gallstone disease have yet to be clarified.

NEW FINDINGS

Hepatic HIF1A promotes cholesterol gallstone formation via hepatic AQP8 suppression and decreased water secretion from hepatocytes. Human studies demonstrate hepatic HIF1A activation in NAFLD patients with gallstones.

LIMITATIONS

Human bile was not analyzed in this study.

IMPACT

Hepatic HIF1A plays a key role in the formation of cholesterol gallstones associated with hepatic steatosis.

disease would be highly beneficial both clinically and economically. Epidemiologically, nonalcoholic fatty liver disease (NAFLD) is known to be associated with gallstones.[4,5] However, the mechanistic links between NAFLD and gallstone disease have yet to be clarified.

Hypoxia-inducible factor 1 (HIF1) is a key transcription factor for adaptive responses to hypoxic conditions and regulates the expressions of various genes involved in oxygen delivery, cellular growth, and redox homeostasis in many tissues and organs.[6,7] HIF1 is a heterodimeric factor consisting of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit.[8,9] The mRNA expression level of *Hif1α* is up-regulated under hypoxic conditions.[8] In addition, the protein level of the HIF1α subunit (HIF1A) is altered dramatically in response to tissue oxygen concentrations. In a state of normoxia, HIF1A rapidly is hydroxylated and bound by von Hippel-Lindau protein, resulting in proteasomal degradation.[10,11] Under hypoxic conditions, HIF1A escapes hydroxylation and forms a dimer with the HIF1β subunit, followed by translocation to the nucleus, and then activates its target genes, including heme oxygenase-1 (*Hmox1*) and vascular endothelial growth factor α (*Vegfa*).[12,13]

In the liver, small amounts of HIF1A protein are detected in perivenous areas because these areas are physiologically hypoxic.[14] In liver steatosis, swelling of hepatocytes caused by lipid accumulation results in decreased hepatic sinusoidal perfusion and impairs hepatic microcirculation, thereby accelerating hepatic hypoxia.[15] In fact, hepatic HIF1A is activated in rodent models of diet-induced liver steatosis[16] and in human beings with NAFLD.[17] Therefore, we sought to elucidate the link between hepatic HIF1A activation and gallstone formation.

Inducible hepatocyte-selective HIF1A knockout markedly suppressed cholesterol gallstone formation and gallbladder inflammation. Hepatic HIF1A deficiency reduced biliary lipid concentrations with increased bile flow rates and up-regulated expression of aquaporin-8 (AQP8), a water channel that is responsible for water secretion from hepatocytes into the bile canalicular lumen.[18] Furthermore, hepatic expressions of *HIF1A* and its downstream targets were increased in human NAFLD patients with gallstones, suggesting the importance of HIF1A expression/activity for gallstone formation in human beings as well. Our results indicate a pivotal role for hepatic HIF1A up-regulation in gallstone formation and suggest that manipulating hepatic hypoxia and/or the resultant bile condensation might be a therapeutic target for cholelithiasis associated with NAFLD.

Materials and Methods

Animal Experiments

Mice with the *Hif1a* allele with a floxed exon 2 were purchased from Jackson Laboratory (Bar Harbor, ME).[19] The mice were housed individually with a 12-h light-dark cycle and controlled ambient temperature of 22°C–25°C. To obtain hepatocyte-selective HIF1A knockout mice, recombinant adenovirus coding the Cre recombinase gene under the control of the CAG promoter was used as previously described.[20–22] Recombinant adenovirus coding the LacZ gene[23] was used as a control. Ten-week-old body weight–matched male *Hif1a*-flox/flox mice were fed ad libitum either a standard laboratory chow diet (65% carbohydrate, 4% dairy fat, 24% protein) or the cholesterol and cholate-rich diet (CCD; 0.5% cholate, 1% cholesterol, 56% carbohydrate, 10% dairy fat, 18% protein, and Oriental yeast, Tokyo, Japan) for 2 weeks. Next, 8-week-old body weight–matched male *Hif1a*-flox/flox mice were injected with adenovirus at a dose of 1.0×10^8 plaque-forming units via the tail vein. Two weeks after adenovirus injections, the diet was switched from a standard laboratory chow diet to a CCD, and mice were fed the CCD for another 1 or 2 weeks. Mice were killed after an 8-hour fast.

Biliary Lipid Studies

Total bile acid, cholesterol, and phospholipid concentrations were determined by enzymatic assays using commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Cholesterol saturation index values were calculated from the critical tables.[24] The concentrations of each of the taurine-conjugated bile acids were determined by liquid chromatography/mass spectrometry (6460 Triple Quadrupole liquid chromatography/mass spectrometry system/1260 Infinity liquid chromatography system; Agilent Technologies, Santa Clara, CA), comparing retention times with bile salt standards. Hydrophobicity index values were calculated as previously reported.[25]

Gallbladder and Gallstone Examinations

Gallbladders were weighed after cholecystectomy. Walls were incised and the contents were expressed onto glass slides or Eppendorf tubes followed by measurement of residual gallbladder wall weights. Gallstone formation was evaluated macroscopically and confirmed by polarized-light microscopy. For each gallbladder, wall thickness was calculated according to the mean value of 6 determinations in the fundic area, as previously reported.[26]