Independent Repression of Bile Acid Synthesis and Activation of c-Jun N-terminal Kinase (JNK) by Activated Hepatocyte Fibroblast Growth Factor Receptor 4 (FGFR4) and Bile Acids*

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The fibroblast growth factor (FGF) receptor complex is a regulator of adult organ homeostasis in addition to its central role in embryonic development and wound healing. FGF receptor 4 (FGFR4) is the sole FGFR receptor kinase that is significantly expressed in mature hepatocytes. Previously, we showed that mice lacking mouse FGFR4 (mR4/−/−) exhibited elevated fecal bile acids, bile acid pool size, and expression of liver cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme for canonical neutral bile acid synthesis. To prove that hepatocyte FGFR4 was a negative regulator of cholesterol metabolism and bile acid synthesis independent of background, we generated transgenic mice overexpressing a constitutively active human FGFR4 (CahR4) in hepatocytes and crossed them with the FGFR4-deficient mice to generate CahR4/mR4/−/− mice. In mice expressing active FGFR4 in liver, fecal bile acid excretion was 64%, bile acid pool size was 47%, and Cyp7a1 expression was 10–30% of wild-type mice. The repressed level of Cyp7a1 expression was resistant to induction by a high cholesterol diet relative to wild-type mice. Expression of CahR4 in mR4/−/− mouse livers depressed bile acid synthesis below wild-type levels from the elevated levels observed in mR4/−/−. Levels of phosphorylated c-Jun N-terminal kinase (JNK), which is part of a pathway implicated in bile acid-mediated repression of synthesis, were 30% of wild-type levels in mR4/−/− livers, whereas CahR4 livers exhibited an average 2-fold increase. However, cholate still strongly induced phospho-JNK in mR4/−/− livers. These results confirm that hepatocyte FGFR4 regulates bile acid synthesis by repression of Cyp7a1 expression. Hepatocyte FGFR4 may contribute to the repression of bile acid synthesis by JNK signaling but is not required for activation of JNK signaling by bile acids.

The fibroblast growth factor (FGF) receptor complex, comprised of oligomeric combinations of transmembrane tyrosine kinase (FGFR), heparan sulfate, and activating FGF, is a ubiquitous regulator of development and adult tissue homeostasis through mediation of cell-to-cell communication and sensing of environmental perturbations (1, 2). The tripartite family is characterized by 22 distinct FGF ligands, four transmembrane receptor kinases (FGFR1-FGFR4), and undefined oligosaccharide motifs in the pericellular matrix. Despite the ubiquitous presence of members of the family, cell-specific expression of FGF, FGFR isotype, and oligosaccharide motifs that interact with FGF ligands and FGFR independently and concurrently combine to confer cell and tissue specificity of FGF signaling (1–4). By far, the most studies on the role of the FGF family have been on cell growth and differentiation in embryogenesis and wound repair in adults (4). Fewer studies have been reported on the role of the family in adult parenchymal organ homeostasis. An increasing number of reports have begun to address the role of the family in adult tissue homeostasis and the disruption of homeostasis that occurs during progression to malignancy and other pathologies (5, 6).

Of the four FGFR kinases, only the genetic ablation of FGFR4 in mice has no overt effect on tissue development, which indicated potential roles limited to adult organ homeostasis rather than development (7). Liver is a central organ in maintenance of adult homeostasis (8). In the normal adult liver, FGFR4 is the sole isofrom of the FGFR kinases that is significantly expressed in mature hepatocytes (9, 10). FGFR4 is most apparent in large hepatocytes adjacent to the central vein but is also expressed in small hepatocytes throughout the lobule (9, 10). We have shown previously in FGFR knock-out mice that FGFR4 does not play a critical role in liver development or adult hepatocyte proliferation in response to loss of liver mass or damage (11, 12). Instead, mice lacking FGFR4 exhibited an elevated excretion of bile acids, bile acid pool, and expression of liver cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme for classical bile acid synthesis. For the first time this indicated a role of the FGF family in regulation of a metabolic function in differentiated adult parenchymal tissue by influencing liver cholesterol metabolism and bile acid synthesis (11). A subsequent study further revealed an increased sensitivity of livers in FGFR4-deficient mice to toxic insult (CCl4). This was coincident with a delay in down-regulation of CYP2E1, the enzyme responsible for generation of toxic metabolic products from CCl4 (12).

The physiological activating FGF ligands and their origin for cholesterol 7α-hydroxylase; CYP27A, sterol 27α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; SHP, short heterodimer partner; LRH-1, liver receptor homologue-1; RIP140, receptor-interacting protein 140; FXR, farnesoid X receptor; JNK, c-Jun N-terminal kinase.

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The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; CahR4, constitutively active human FGFR4; CYP7A1, cholesterol 7α-hydroxylase; CYP27A, sterol 27α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; SHP, short heterodimer partner; LRH-1, liver receptor homologue-1; RIP140, receptor-interacting protein 140; FXR, farnesoid X receptor; JNK, c-Jun N-terminal kinase.

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Hepatocyte FGFR4 and Bile Acid Synthesis

Generation of Transgenic Mice Expressing Wild-type and Constitutively Active Human FGFR4 (CahR4) in Liver—Full-length human FGFR4 cDNA was cloned from HepG2 cells. Mutant human FGFR4 cDNA coding for glutamate instead of lysine 645 in the autoinhibition loop of the FGFR4 tyrosine kinase was generated by PCR-mediated site-directed mutagenesis using the human FGFR4 cDNA template. The wild-type or mutant human FGFR4 cDNA was inserted downstream of a 2.3-kb albumin promoter obtained from Dr. S. Thorgeirsson (NCI, National Institutes of Health) in the pSK-ALB/SV40 vector. A chicken insulator was inserted at the 3′-end of the transgene at the XhoI site. The ALB-hR4 or ALB-CahR4 transgene was then excised with BssHII restriction enzyme and purified for pronuclear microinjection. Procedures for generation of transgenic mice have been described in detail elsewhere (20, 21). Genomic DNA from tails of founder mice was digested with EcoRI and analyzed by Southern blot hybridization with a human FGFR4 cDNA probe.

Hepatocytes, Riboprobes, and Analysis of mRNA—Full-length cDNAs coding for murine Cyp7a1 (pCMV-mCyp7a1), CYP7B1 (pCYP7B1), and CYP27A1 (pCMVM270H) were gifts from Dr. David W. Russell (University of Texas Southwestern Medical Center at Dallas, Dallas, TX). Full-length human FGFR4 cDNA was cloned from HepG2 cells. Murine SHP cDNA was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from mouse liver using sense primer 5′-TCTTACGC-GAGATTGAAGC-3′ and antisense primer 5′-AACGAGATGGC-CAGTGAG-3′. Murine CYP8B1 cDNA was amplified by RT-PCR from mouse liver using sense primer 5′-ATGACGTGGTCAAGAAATGCT-3′ and antisense primer 5′-TGTTCTACTGAGGATAGCC-3′. Murine β-actin, FGFR4, and RIP140 cDNAs were described previously (11). Riboprobes complementary to parts of the cDNAs described above that had been subcloned into pBluescript-SK were transcribed into 32P-labeled antisense riboprobes by T3 or T7 RNA polymerase using the MAXiscript kit (Number 11236; Ambion, Austin, TX). The size of the probes and the predicted protected fragments, respectively, for the following mRNAs were: β-actin, 197 and 139 nt; murine CYP4F4, 267 and 198 nt; human FGFR4, 268 and 248 nt; Cyp7a1, 272 and 219 nt; Cyp27a, 318 and 268 nt; Cyp7b1, ~200 and ~170 nt; SHP, 261 and 248 nt; LRH-1, 354 and 280 nt; Cyp8b1, 236 and 222 nt; RIP140, 306 and 226 nt. Total RNA was extracted from liver samples with the Tri-reagent RNeasy kit (Number BL-10200; Biotexc Laboratories, Houston, TX), and specific mRNAs were measured by ribonuclease protection assay (RPA) using the Hyb-Speed RPA kit (Number 11412; Ambion). About 50 μg of liver RNA was hybridized with 1 × 10^6 cpm of 32P-labeled specific antisense and β-actin riboprobes in the same reaction mixture. After treatment with ribonuclease, protected products were analyzed on 5% polyacrylamide sequencing gels, followed by autoradiography. Size of protection products was determined from the product of a DNA sequencing reaction parallel to the protection assays. The amount of each radiographic product was quantified using a Phosphorimager (Amersham Biosciences) and normalized to bands in the same sample. Experimentally expressed bands in units relative to the level of expression in a wild-type mouse on standard chow that was assigned a value of one as described in the figure legends.

Histological and Immunohistochemical Analyses—Liver tissue sections were prepared and analyzed as described previously (11). For analysis of proteins, livers were homogenized in phosphate-buffered saline containing 0.5% sodium deoxycholate, 0.1% SDS, 1.0 mm NaF, 1.0 mm Na3PO4, and 1.0 phenylmethylsulfonyl fluoride, and the solubilized extract was clarified by centrifugation. Protein concentration was determined using the BCA protein assay reagent (Number 23225X; Pierce, Rockford, IL). A total of 100 μg of soluble liver protein was incubated with a 1/1000 dilution of rabbit anti-JNK antibody (Cell Signaling Technology, Beverly, MA) that recognizes both phosphorylated and unphosphorylated protein and immunoprecipitated by protein A-agarose beads. The immunoprecipitate was then subjected to 10% SDS-PAGE and transferred to Hybond-P membrane (Amersham Biosciences), which was incubated with a 1/2000 dilution of mouse anti-phospho-JNK antibody (Cell Signaling Technology). This was followed by washing and incubation with a 1/20000 dilution of goat anti-rabbit antibody coupled to horseradish peroxidase (Bio-Rad). Bands were visualized by development with ECL-Plus detection reagents (Amersham Biosciences) and quantitated using an AlphaImager (Alpha Innotech, San Leandro, CA). The same membrane was stripped and then incubated with 1/2000 dilution of rabbit anti-JNK antibody (Cell Signaling Technology). Experimental values were expressed in arbitrary densitometric units relative to the level of expression in a wild-type mouse that was assigned a value of one.

**EXPERIMENTAL PROCEDURES**

**Animals and Diets**—Mice lacking FGFR4 have been described (7). Adult FVB mice were purchased from Charles River (Wilmington, MA), and young adult Swiss/Webster mice were purchased from Harlan Sprague-Dawley (Houston, TX). Adult mice 7–8 weeks old were used in the study. Mice were maintained in 12 h of light/12 h of dark cycles and were given free access to food and water. Standard rodent chow containing 0.02% (w/w) cholesterol, standard chow supplemented with 2% (w/w) cholesterol, and standard chow supplemented with 0.5% (w/w) cholesterol were obtained from Alief Purina Feed Store, Inc. (Alief, Texas). Three to eight mice were employed per experimental group, as described in specific figure legends. Mice were weighed, anesthetized, and exsanguinated, and the livers or other tissues were harvested at 10 a.m. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at the Institute of Biosciences and Technology, Texas A&M University System Health Science Center.

FGFR4 in the liver have not been established. Complexes of recombinant FGFR4 and heparin bind both FGF1 and FGF2 in vitro but only FGF1 in the presence of hepatocyte heparan sulfate (9). Human FGFR19, of which FGF15 is the mouse counterpart (13, 14), has been reported to bind only recombinant FGFR4 in vitro (15). Structure-based molecular modeling has supported arguments that FGF19 might be a specific activating ligand for FGFR4 (16). Overexpression of FGFR4 in transgenic mice and applications of recombinant FGFR19 intravenously or at neutral sites increased metabolic rate and caused decreased body weight, adiposity, and dietary diabetes (5, 17). Although bile acid pools were not measured, the treatment reduced expression of Cyp7a1 while exerting both positive and negative effects on expression of multiple genes involved in cholesterol metabolism. Moreover, it has been shown that agonists of the bile acid-activated transcription factor farnesoid X receptor (FXR) induced expression of FGFR19 at the transcription level in primary cultures of human hepatocytes concurrent with repression of CYP7A1 (18). In addition, the treatment of cultured hepatocytes with FGFR19 activated the JNK signaling pathway that is activated by treatment of cultured hepatocytes with bile acids; this was accompanied by repression of CYP7A1 and bile acid metabolism (19). Although the authors did not examine the effect of other added members of the FGF ligand family on hepatocytes, FGFR19 was the single FGF most significantly induced by application of the FXR agonist. These results suggest that there is potentially a local autocrine loop of FXR-induced FGFR19 acting through hepatocyte FGFR4 that activates JNK signaling and that this might mediate feedback control of cholesterol to bile acid metabolism in the liver.

Here we confirmed that hepatocyte FGFR4 is a negative feedback mediator of bile acid synthesis in the liver independent of its physiological activating FGF. We expressed a constitutively active FGF-independent human FGFR4 in mouse hepatocytes by gene targeting using the albumin promoter. The transgenic mice exhibited decreased fecal bile acid content, a decreased circulating bile acid pool, and depressed expression of liver Cyp7a1 concurrent with the elevation of phosphorylated c-Jun N-terminal kinase (JNK). Progeny (CahR4/mR4/−/−) of mice bearing constitutively active human FGFR4 (CahR4) were mated with the FGFR4-deficient mice (mR4/−/−), and they exhibited bile acid pools and levels of phosphorylated JNK similar to the CahR4 parent. This indicated that hepatocyte FGFR4 regulates bile acid synthesis independent of mouse background. However, 0.5% cholate in the diet similarly stimulated JNK phosphorylation in the FGFR4-deficient mice. This, and our previous observation that the elevated Cyp7a1 expression in mR4/−/− mice is still subject to repression by dietary cholate, suggests that although both activated FGFR4 and bile acids activate the JNK pathway they control bile acid synthesis by independent mechanisms.

**Animals and Diets**—Mice lacking FGFR4 have been described (7). Adult FVB mice were purchased from Charles River (Wilmington, MA), and young adult Swiss/Webster mice were purchased from Harlan Sprague-Dawley (Houston, TX). Adult mice 7–8 weeks old were used in the study. Mice were maintained in 12 h of light/12 h of dark cycles and were given free access to food and water. Standard rodent chow containing 0.02% (w/w) cholesterol, standard chow supplemented with 2% (w/w) cholesterol, and standard chow supplemented with 0.5% (w/w) cholesterol were obtained from Alief Purina Feed Store, Inc. (Alief, Texas). Three to eight mice were employed per experimental group, as described in specific figure legends. Mice were weighed, anesthetized, and exsanguinated, and the livers or other tissues were harvested at 10 a.m. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at the Institute of Biosciences and Technology, Texas A&M University System Health Science Center.
Results

Depressed Bile Acid Synthesis in Mice Expressing Constitutively Active Hepatocyte FGFR4—We first prepared transgenic mice overexpressing wild-type FGFR4 in the liver under control of the hepatocyte-specific albumin promoter. Use of the albumin promoter has resulted in liver-specific phenotypes with the c-myc, transforming growth factor α, and hepatocyte growth factor genes (22). FGFR4 cDNA was inserted in vector pSE-ALB/SV40 comprised of a 2.3-kb albumin promoter, an RNA splice site, the poly(A) addition site from SV40 T antigen, and an insulator element from the 5’ region of the chicken β-globin locus (Fig. 1A). Transgenic animals were identified by Southern blot. Ribonuclease protection analysis of liver mRNA indicated that expression of the human FGFR4 transgene was significant, but we detected no differences in fecal bile acid content or bile acid pools in two different transgenic lines from those of control wild-type littermates.

Because the overexpressed wild-type FGFR4 may be limited by an activating GF, we generated transgenic mice overexpressing a constitutively active human FGFR4 mutant (K645E) in which lysine 645 in the autoinhibition loop of the FGFR4 tyrosine kinase domain was substituted by glutamate. An autosomal dominant mutation at the homologous site in the human FGFR3 gene occurs naturally and causes developmental defects that are due to gain of function and FGF-independent tyrosine kinase activity (23). The FGFR4 (K645E) mutant tyrosine kinase, when targeted to the plasma membrane by a myristoylation signal transformed NIH3T3 cells, induced FGF-independent neurite outgrowth in PC12 cells, and stimulated phosphorylation of downstream targets SHP2, PLC-γ, mitogen-activated protein kinase, and phosphatidylinositol-3 kinase activity (24). Two transgenic lines referred to as CahR4 mice were generated that expressed the FGFR4 (K645E) construction in liver (Fig. 1). Analysis of human FGFR4 mRNA from various tissues of the CahR4 mice with both human and mouse FGFR4 cRNA riboprobes indicated that human FGFR4 was expressed only in the liver (Fig. 1C). Similar to the transgenic mice bearing wild-type FGFR4, both lines appeared normal at birth and exhibited no signs of abnormalities from postnatal development to adulthood. Morphological and histological analysis revealed no gross differences in the livers or gallbladders of CahR4 transgenic mice from their wild-type littermates (data not shown). In contrast to the FGFR4−/− mice (11), the size of gall bladders was normal and fecal bile acid levels and total bile acid pools were decreased in the CahR4 mice (Fig. 2, A and B). Fecal bile acids fell from 9 to 5.8 μmol/day/100 g of body weight (p < 0.001), and the total bile acid pool decreased from 70 to 33 μmol/100 g of body weight (p < 0.002). The bile acid pool in CahR4 mice was an average of 60% that of wild-type in newborns through the weaning spike of bile acid production and on into adulthood (Fig. 2C).

To explore the cause of the decrease in fecal bile acids and bile acid pools in the CahR4 mice, we compared the expression of several key and secondary gene products involved in bile acid homeostasis at the mRNA level in the livers of CahR4 and FGFR4−/− mice (11) to those from wild-type controls fed normal chow (Fig. 3). Endogenous levels of mouse FGFR4 were constant in both wild-type and CahR4 transgenic animals (Fig. 3A). Levels of mRNA for CYP7A1, the rate-limiting enzyme in the classical route of bile acid synthesis, were reduced to 10–30% of that in wild-type livers concurrent with the observed reduced bile acid levels in the animals (Fig. 3B).

No change from wild-type levels in expression of Lrh-1 (liver receptor homologue-1) with expression of constitutively active FGFR4 in CahR4 mice (Fig. 3C). LHR-1 is an orphan nuclear receptor involved in the liver-specific transcription of Cyp7a1 (25). LHR-1 is antagonized by SHP (short heterodimer partner), a non-DNA binding nuclear receptor that is induced by bile acids through FXR. This has emerged as one pathway by which bile acids repress CYP7A1 (26–28). The expression of Shp was decreased to 30% of wild-type in CahR4 livers (p < 0.001) (Fig. 3D). However, there was no significant change in FGFR4−/− livers (p > 0.05) (Fig. 3E) despite the significant elevation of bile acid pool size (11). Similar to the livers of FGFR4−/− mice (11), levels of mRNA for CYP27A and CYP7B1 were unchanged from wild-type in CahR4 mice (Fig. 3, E and F). Both enzymes are involved in secondary alternative pathways to the classical route of bile acid synthesis. We also examined mRNA levels of CYP8B1 (sterol 12α-hydroxylase) that is involved in the classical route of bile acid synthesis downstream of CYP7A1 and is subject to some of the same regulatory inputs (26, 29). Compared with wild-type controls, the expression of Cyp8b1 was unchanged in CahR4 livers (p > 0.6) but increased 4-fold in FGFR4−/− livers (p < 0.03) (Fig. 3G).

Lastly, no change in mRNA levels for RIP140 (receptor interacting protein 140) was observed in the CahR4 livers.

Fig. 1. Overexpression of a constitutively active human fibroblast growth factor receptor 4 (CahR4) in the liver. A, schematic of the CahR4 transgene. The mutant human FGFR4 cDNA (K645E) was inserted downstream of a 2.3-kb albumin (ALB) promoter. A chicken insulator was inserted at the 3’-end of the transgene. B, genomic integration of the CahR4 transgene. Tail genomic DNA of wild-type (WT) and founder (CahR4) mice was digested with EcoR1 and analyzed by Southern hybridization with a human FGFR4 cDNA (hR4) probe. M, markers; C, liver-specific expression of the human CahR4 transgene. Expression of both human (hR4) and mouse (mR4) FGFR4 mRNA was analyzed from total RNAs with human FGFR4 and mouse FGFR4 cRNA riboprobes. RNA from livers from wild-type (lane 1) and transgenic mouse (lane 2) livers, as well as transgenic mouse heart (lane 3), lung (lane 4), kidney (lane 5), stomach (lane 6), small intestine (lane 7), and spleen (lane 8), was analyzed. P, probe; Y, yeast tRNA.
compared with wild-type (Fig. 3H), although Rip140 expression was significantly decreased in the FGFR4-/- livers (11). Previously we suggested that the loss of FGFR4-dependent up-regulation of expression of Rip140, a candidate repressor of liver receptor X, explained the hepatomegaly observed in FGFR4-/- mice fed a high cholesterol and cholate diet. Liver Rip140 is down-regulated by high dietary cholesterol but up-regulated by dietary cholate (11). The lack of an increase in RIP140 in the CahR4 mice may result from an opposing effect on RIP140 due to decrease in the bile acid pool. Alternatively, FGFR4 may not regulate Rip140 expression directly. Our previous results may reflect a decrease in Rip140

**Fig. 2.** Decreased fecal bile acids and bile acid pools in mice expressing constitutively active human FGFR4 in the liver. A, fecal bile acid excretion. Excreted bile acids of eight wild-type and eight CahR4 mice were compared. The data are the mean ± S.D., and the difference significant at $p < 0.001$ (Student's t test). B, decrease in bile acid pools in adult CahR4 mice. Data are the mean ± S.D. of eight mice with significant difference at $p < 0.002$. C, decrease in bile acid pools during postnatal development. Values are the means ± S.D. of three mice at each time point. Significant difference between wild-type and CahR4 at each time point was $p < 0.05$. Bile acids were determined as described under “Experimental Procedures.”

**Fig. 3.** Expression of genes involved in bile acid metabolism in CahR4 and FGFR4-/- mice. Expression of the CahR4 transgene (hR4) and mouse FGFR4 (mR4) (A), CYP7A1 (B), LRH-1 (C), SHP (D), CYP27A (E), CYP7B1 (F), CYP8B1 (G), and RIP140 (H) mRNA was determined by ribonuclease protection assay as described under “Experimental Procedures” using 50 μg of total RNA isolated from individual livers. Change in the expression of genes relative to β-actin controls is indicated, with the expression in wild-type mouse liver assigned a value of 1. Densitometric quantitation was performed as described under “Experimental Procedures.” Significance of differences were as follows: WT and CahR4 CYP7A1 $p < 0.01$ ($n = 4$); WT and CahR4 LRH-1 $p = 1$, WT and FGFR4-/- $p > 0.1$ ($n = 3$ or 4); WT and CahR4 SHP $p < 0.001$, WT and FGFR4-/- SHP $p > 0.05$ ($n = 3$ or 4); WT and CahR4 CYP27A $p = 1$ ($n = 3$ or 4); WT and CahR4 CYP8B1 $p = 1$ ($n = 3$ or 4); WT and CahR4 CYP8B1 $p > 0.6$, WT and FGFR4-/- CYP8B1 $p < 0.03$ ($n = 3$ or 4); WT and CahR4 RIP140 $p = 1$, WT and FGFR4-/- RIP140 $p < 0.03$ ($n = 3$ or 4).
ences in CahR4 mice were not significant except for the expression in wild-type animals on normal diet is indicated. Active FGFR4 and its ablation in FGFR4 reciprocal regulation by the overexpression of constitutively active FGFR4 and bile acid synthesis, fecal bile acids. An increase in the total bile acids in those fed normal chow (Fig. 4, p0.04, respectively). CYP7A1 mRNA was determined as in Fig. 3 from a pool of liver RNA from three of the animals. The change relative to expression in wild-type animals on normal diet is indicated.

expression predominantly because of the increased cholesterol input because cholesterol synthesis is increased in the FGFR4−/− mice. In summary, the results of this series indicate that only Cyp7a1 expression exhibited the expected reciprocal regulation by the overexpression of constitutively active FGFR4 and its ablation in FGFR4−/− that was coincident with the depression and elevation of bile acid pools in the two mouse strains, respectively.

Dietary Cholesterol Fails to Increase Bile Acid Pools in CahR4 Transgenic Mice—Wild-type mice respond to high cholesterol in the diet by increasing bile acid synthesis, fecal bile acid excretion, and the total bile acid pool (11, 30). We determined the response of the CahR4 mice to dietary cholesterol by comparison to wild-type mice when both were fed 2% cholesterol (w/w) for 21 days. As expected, the fecal bile acids in the wild-type mice fed high cholesterol were three times that of those fed normal chow (Fig. 4A). An increase in the total bile acid pool was not as marked but still significant (p<0.05) (Fig. 4B). Cholesterol-fed CahR4 mice failed to exhibit a statistically significant increase (p>0.5) over the severely depressed levels of either fecal bile acids or the total bile acid pool (Fig. 4, A and B). An increase over the depressed mRNA levels of CYP7A1 in CahR4 mice was observed in mice fed high cholesterol, but CYP7A1 mRNA levels remained half that observed in wild-type mice on a regular diet (Fig. 4C). Analysis of hepatic cholesterol levels indicated they were similar in both wild-type and CahR4 mice on normal chow (Fig. 4D). Although the high cholesterol diet caused an expected 1.5-fold increase in hepatic cholesterol concentration in wild-type mice, the increase in CahR4 livers was modest (p=0.04) (Fig. 4D). This was unexpected because the CahR4 repression of CYP7A1 levels should inhibit disposal of liver cholesterol to bile acids and cause an even greater accumulation of liver cholesterol. The results may indicate that the bile acid deficiency caused by FGFR4 hyperactivity is sufficiently severe to limit intestinal uptake of cholesterol in quantities sufficient to overload the liver.

Progeny of CahR4 x FGFR4−/− Mice Exhibit Repressed Levels of Bile Acid Synthesis—FGFR4−/− mice exhibited 3 and 1.7 times the fecal bile acid content and total bile acid pool size, respectively, of that exhibited by wild-type mice (Fig. 5). To further confirm that it is the absence of activated FGFR4 that caused the elevation, we mated the CahR4 mice with the FGFR4−/− mice and then compared the fecal bile acid excretion and bile acid pools in the progeny (WT, FGFR4−/−, and CahR4/R4−/−) and parent strains. The CahR4/R4−/− hybrids exhibited a reversal of the elevated bile acid levels observed in the FGFR4−/− parent to below the normal levels observed in wild-type mice (Fig. 5). Fecal bile acid content was reduced to about the levels observed in the CahR4 parent and to 15% of those of the FGFR4−/− littersmates (Fig. 5A). The total bile acid pools in the CahR4/R4−/− hybrids were reduced to those of the CahR4 parent and to 30% of the FGFR4−/− littersmates (Fig. 5B). Fecal bile acid content and total pools in progeny were similar to those of the FGFR4−/− parent (data not shown) (11). Consistent with the changes in fecal bile acids and bile acid pool size, the expression of Cyp7a1 was significantly elevated in FGFR4−/− littersmates (p<0.02) from the cross but significantly decreased in the CahR4/R4−/− littersmates (p<0.04) (Fig. 5C). These results indicate that the CahR4 strain was dominant over the FGFR4−/− strain. The constitutively active hepatocyte FGFR4 transgene completely reversed the abnormal elevation caused by the absence of mouse FGFR4. These results further suggest that the regulation of bile acid synthesis by FGFR4 is not limited to either the FVB strain used for the transgenic progeny of CYP7A1 mRNA by bile acids and JNK in mouse livers that were deficient in FGFR4 and those expressing constitutively active FGFR4. Phospho-JNK levels in the FGFR4−/− livers were 30% of wild-type livers, whereas the hybrid CahR4/R4−/− livers exhibited an average 1.8-fold increase over the wild-type livers (Fig. 6A). This indicated that FGFR4 activates the JNK signaling pathway in hepatocytes and, thus, may contribute to cholesterol and bile acid homeostasis by the pathway under normal dietary conditions. To determine whether FGFR4 was required for the bile acid-stimulated activation of JNK, we fed FGFR4−/− mice a 0.5% cholate diet for 7 days and examined the level of phospho-JNK in liver homogenates (Fig. 6B). Despite the absence of FGFR4, dietary cholate caused an increase in activated JNK of 3- to 5-fold (Fig. 6B). Thus, artificially high bile acids activate the JNK pathway independent of FGFR4. FGFR4 or its ligands are not obligatory for the stimulation of the JNK pathway by elevated levels of bile acids.

DISCUSSION

Here we used hepatocyte-specific gene targeting in mice to confirm that activated hepatocyte FGFR4 is a negative regulator of bile acid synthesis and, potentially, cholesterol metabolism. This confirms our conclusion based on the observation that bile acids were elevated in mice in which FGFR4 was
ablated from the genome entirely. To bypass the requirement for an activating ligand whose identity is currently unknown, an FGFR4-independent mutant of FGFR4 (CahR4) in which the tyrosine kinase was constitutively active was overexpressed specifically in adult hepatocytes. The CahR4 mouse phenotype was similar to that of adult Cyp7a1-deficient mice that exhibited decreased fecal bile acids and total bile acid pool (33, 34). Despite the suppressed level of bile acids, the CahR4 mouse pups developed normally with a high survival rate. This was in contrast to Cyp7a1-deficient pups, of which 90% died during the first 18 days of postnatal development unless given a supplement of fat-soluble vitamins and cholate (33). This is consistent with our results that show that CYP7A1 mRNA is not suppressed to zero in CahR4 pups, and therefore bile acid levels are sufficient to prevent lethal vitamin- and bile acid-related deficiencies during the critical window of development when non-CYP7A1-dependent bile acid pathways are undeveloped.

The ability of high dietary cholesterol to detectably up-regulate Cyp7a1 expression in the CahR4 mice also suggested that sufficient bile acids were produced to support sufficient intestinal uptake of cholesterol to maintain CYP7A1 by induction. This further indicated that the liver X receptor activation system was intact in the CahR4 mice and qualitatively unimpaired by the constitutive activity of FGFR4. We cannot completely rule out that hyperactivity of FGFR4 contributes to the quantitative repression of inductive mechanisms of CYP7A1 because there may be limitations in cholesterol uptake due to bile acid deficiency caused by the Cyp7a1 repression. The fact that cholesterol fails to accumulate in livers of the CahR4 mice fed high cholesterol even at severely reduced CYP7A1 levels suggests there may be limitations in cholesterol uptake caused by the bile acid deficiency. A comparison of cholesterol uptake in the CahR4 mice compared with wild-type is needed to clarify this question.

A comparison of CYP7A1 mRNA levels to several other enzymes and regulators involved in bile acid synthesis in CahR4 and FGFR4/H11002/H11002 mice revealed that only CYP7A1 mRNA was clearly reciprocally down-regulated and up-regulated coincident with the decreased and increased bile acid levels in the two mouse strains, respectively. This confirms that the regulation of bile acid synthesis by hepatocyte FGFR4 results primarily from the regulation of CYP7A1 activity at transcription, although the mode of FGFR4 regulation from membrane-bound activation.
Hepatocyte FGFR4 and Bile Acid Synthesis

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tyrosine kinase to Cyp7a1 transcription remains obscure. No effect of FGFR4 on the critical liver Cyp7a1 transcriptional regulator LRH-1 was observed. Our results also suggest that FGFR4 does not modify CYP7A1 levels and bile acid pools by direct effects on expression of FXR-induced LRH-1 antagonist SHP in the two mouse strains. SHP mRNA levels decreased along with the decrease in bile acid pools in the mice with hyperactive liver FGFR4 but did not increase in response to elevated bile acids in the FGFR4-deficient mice (11). These results are puzzling because hyperactive FGFR4 increases activated JNK and up-regulation of SHP expression is thought to be responsive to activated JNK (19, 32). The depression of SHP likely is an indirect consequence of the depression in bile acids (35) caused independently by the hyperactive FGFR4. The presence or absence of FGFR4 had no effect on expression of enzymes involved in secondary alternative pathways to the classical route of bile acid synthesis. Hyperactive liver FGFR4 did not impact levels of CYP8B1 mRNA, an enzyme in the classical route of bile acid synthesis downstream of CYP7A1 that is subject to some of the same controls (26, 27). However, Cyp8b1 expression increased 4-fold in FGFR4-deficient livers, indicating that similar to CYP7A1 FGFR4 may also play a role in maintenance of steady-state levels of CYP8B1 under normal dietary conditions.

FGF family signaling largely mediates local cell-to-cell communication and plays roles in both development and adult organ homeostasis as a sensor of perturbations in the immediate local environment (1, 2). This generally elicits a local cellular response that usually results in restoration or remodeling of the tissue environment. Although some models accommodate FGF-independent activation of the FGFR complex upon perturbation of the pericellular matrix or membrane, activation is thought to generally occur by release of an activating FGF from neighboring cells or pericellular matrix reservoirs. In line with this, we have proposed previously that the primary role of hepatocyte FGFR4 is as a sensor of damage or infection in the local liver environment that is linked to liver-specific metabolic homeostasis and metabolite-controlled transcriptional networks such as cholesterol and bile acid (11) and xenobiotic (12) metabolism. This view predicts that the impact of hepatocyte FGFR4 on cholesterol and bile acid metabolism that we observe in the genetically altered animals reflects a local role of FGFR4 in maintenance of liver metabolic homeostasis rather than a major role in mediation of systemic cholesterol and bile acid balance in response to dietary load.

However, recent findings suggest that two members of the 22-member FGF ligand family, FGF19 and FGFR3, may play endocrine-like roles in metabolic homeostasis. Although a specific organ of origin and specific target cell and receptor have not been identified, circulating FGFR3 dramatically affects phosphate homeostasis in humans (36), and an FGFR3 mutation underlies hypophosphatemic rickets (37). Increased levels of circulating human FGFR3 of which the mouse ortholog is FGFR5 (13) increased metabolic rate without reducing food intake, reduced adiposity, and reversed dietary and leptin-resistant mice (5, 17). Two reports, one based on indirect cell-free interaction analysis between tagged recombinant FGF19 and FGFR isotypes (15) and the other modeling of a complex of FGFR9 and FGFR4 (16), have argued that FGFR9 is exclusively a ligand for FGFR4 and not other isoforms of FGFR.

A role of FGFR1-FGFR4 signaling in liver in systemic regulation of cholesterol metabolism and bile acid synthesis has been further buttressed by the up-regulation of liver Fgf19 transcription by agonists of the FXR bile acid receptor and the presence of an FXR-RXRα binding element in intron 2 of the FGF19 gene (18). FGF19 administered to primary cultures of human hepatocytes (18) or systemically to mice (5, 17, 18) strongly suppressed the expression of Cyp7a1. This occurred independent of the induction of SHP. The authors further demonstrated that the effect of FGF19 on Cyp7a1 expression was mediated by JNK activation (18) that has been implicated in the suppression of Cyp7a1 by bile acids (19). From this it has been proposed that the FXR-FGF19-JNK signal cascade with FGFR4 tyrosine kinase, the mediator for FGF19, is an alternate bile acid-FXR-induced pathway for repression of Cyp7a1. In potential agreement, we showed that levels of activated JNK were reduced in FGFR4−/− mice and restored to normal levels with genetic restoration of active FGFR4. However, bile acids still activated JNK independent of FGFR4. This indicated that FGFR4 is not required for the bile acid- or FGF19-induced repression of Cyp7a1 and bile acid synthesis. Fu et al. (5) recently reported unpublished observations that the effects of FGF19 on metabolic syndrome are intact in FGFR4 knock-out mice. This casts doubt on the contention that FGFR4 is the sole mediator of the biological effects of FGF19. More study is needed to verify that FGF19 is an activator of FGFR4, particularly in the liver context even though it is clear it is not specific for it. The issue of what is the receptor for FGF19 in both bile acid metabolism and metabolic syndrome, as well as what is the activating ligand or mode of activation of FGFR4 in the liver, remains an open question.

Similar to Cyp7a1-deficient mice (34), mice with constitutively active FGFR4 exhibited no increase in fecal bile acids, bile acid pools, or Cyp7a1 expression levels in response to a high cholesterol diet that normally increases bile acid levels and CYP7A1 in wild-type animals (34, 38). Moreover, the livers of Cyp7a1−/− mice do not exhibit hypercholesterolemia and hyperlipidemia due to accumulation of liver cholesterol. These results are consistent with the reported resistance of the mouse to hypercholesterolemia even when bile acid metabolism at the level of CYP7A1 is severely perturbed (11, 39–41). Nonetheless, adaptive conversion of cholesterol to bile acids is thought to play an important role in cholesterol disposal and cholesterol balance in humans (42, 43). A genetic deficiency of Cyp7a1 in humans causes a decrease in bile acid production and accumulation of cholesterol in the liver accompanied by down-regulation of LDL receptors and hypercholesterolemia (44). Overexpression of Cyp7a1 in mouse livers blocked bile acid diet-induced atherosclerosis and gallstone formation (45). Conceivably, the constitutively altered hyperactivity of liver FGFR4 might contribute to diseases of cholesterol and bile acid metabolism in humans.

Autosomal dominant point mutations cause constitutive activation among FGFR genes, resulting in developmental abnormalities in humans (46). However, to date no gain of function mutants in the FGFR4 gene have been characterized. Frequent polymorphisms in coding sequence for the transmembrane domain of FGFR4 have been reported (47). A change from a neutral or hydrophobic amino acid in the transmembrane domain to a charged one by point mutation is a frequent cause of gain of function in other FGFR isotypes. About 55% of humans carry a glycine to arginine change at residue 388 in FGFR4. MDA-MB-231 mammary tumor cells expressing the FGFR4 Arg388 exhibited increased motility relative to cells expressing the FGFR4 Gly388 isotype. The FGFR4 Arg388 allele was also associated with breast cancer progression, early lymph node metastasis, and advanced tumor node metastasis stage in colon cancer patients (47). Our observations suggest that a screen of the population bearing the FGFR4 Arg388 may be of interest to determine whether alterations in FGFR4 may be a risk factor in hypercholesterolemia and whether depression of FGFR4 activity may be an indirect target for enhancing CYP7A1 activity (45).
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