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JNK-mediated disruption of bile acid homeostasis promotes intrahepatic cholangiocarcinoma

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Metabolic stress causes activation of the cjun NH2-terminal kinase (JNK) signal transduction pathway. It is established that one consequence of JNK activation is the development of insulin resistance and hepatic steatosis through inhibition of the transcription factor PPARα. Indeed, JNK1/2 deficiency in hepatocytes protects against the development of steatosis, suggesting that JNK inhibition represents a possible treatment for this disease. However, the long-term consequences of JNK inhibition have not been evaluated. Here we demonstrate that hepatic JNK controls bile acid production. We found that hepatic JNK deficiency alters cholesterol metabolism and bile acid synthesis, conjugation, and transport, resulting in cholestasis, increased cholangiocyte proliferation, and intrahepatic cholangiocarcinoma. Gene ablation studies confirmed that PPARα mediated these effects of JNK in hepatocytes. This analysis highlights potential consequences of long-term use of JNK inhibitors for the treatment of metabolic syndrome.

Significance

Obesity is associated with hepatic steatosis and activation of the cjun NH2-terminal kinase (JNK) stress-signaling pathway. Studies in mice demonstrate that JNK deficiency in the liver prevents the development of hepatic steatosis. This observation suggests that inhibition of JNK signaling may represent a possible treatment for hepatic steatosis. However, the long-term consequences of JNK inhibition are poorly understood. Here we demonstrate that loss of JNK causes changes in cholesterol and bile acid metabolism that promote cholestasis, bile duct proliferation, and intrahepatic cholangiocarcinoma. We identify PPARα activation as the molecular mechanism that accounts for this phenotype. Our analysis has important implications for the long-term use of JNK inhibitors for the treatment of obesity.


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The authors declare no competing interest.

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Data deposition: The RNA-seq analysis (Fig. S2) was performed using a dataset that we have previously published (7) that was deposited in the GEO database (GSE55190).

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Results

Hepatic JNK Deficiency Alters Bile Acid Homeostasis. We have previously shown that hepatic JNK deficiency results in the activation of the transcription factor PPARα and causes protection against diet-induced insulin resistance and hepatic steatosis (7). The activation of PPARα may cause altered BA metabolism (19). We therefore examined BA in hepatic JNK1 plus JNK2-deficient mice (L^DKO) and control mice (L^WT) at 6 mo of age. We found that the total BA concentration in the blood of L^DKO mice was significantly increased compared with L^WT mice (Fig. 1B). Hepatic expression of genes related to hepatic PC synthesis (Scd2, Chpt1, and Chkh) or hepatocyte-mediated transport of PC (Abcb4 and Atp8b1) and BA (Abc11 and Scl10a1) was markedly increased in L^DKO mice (SI Appendix, Fig. S1A and B). Similarly, increased expression of genes related to cholesterol synthesis (Hmgcs1, Hmgcr, and a large increase in liver mass together with significant inflammation and cholangiocarcinoma markers were reduced in LPPARα mice (Fig. 4D). Gene expression analysis demonstrated that both inflammation and cholangiocarcinoma markers were reduced in LPPARα/DKO mice compared with L^DKO mice (Fig. 5A and B). This evidence suggests that PPARα deficiency protected against the promotion of cholangiocyte proliferation in mice lacking hepatocyte JNK1/2. To evaluate whether PPARα deficiency and subsequent normalization of BA production blunted the FXR/FGF15/FGFR4 pathway, we evaluated FXR target gene expression. We found that hepatic expression of Fgf15 and Shp was reduced in LPPARα/DKO mice compared with L^DKO mice (Fig. 5C). Indeed, immunoblot analysis confirmed that PPARα deficiency suppressed the increased FGF15/21 expression detected in JNK-deficient cholangiocytes (Fig. 5D). This is consistent with the observation of lower levels of ERK activation, detected by immunohistochemistry, in LPPARα/DKO cholangiocytes (Fig. 5E).

Discussion

Since increased BA can lead to inflammation, apoptosis, and necrosis of hepatocytes (31, 32), long-term elevated BA levels in patients are considered a risk factor for liver cancer development (33). Indeed, serum BA might be useful for the diagnosis of cholangiocarcinoma (34). Our findings provide an animal model...
in which defects in BA homeostasis are linked to cholangiocarcinoma (Fig. 6).

Previous studies have established that hepatic JNK deficiency can suppress cholangiocyte proliferation and oncogenic transformation in a p53/Kras-induced model of cholangiosarcoma (35) and promotes cholangiocarcinoma in dethylnitrosamine and NEMO deficiency models of liver cancer (36). The results of the present study demonstrate that hepatic JNK deficiency is sufficient for the development of cholangiocyte malignancy (Fig. 2).

PPARα is an important modulator of liver metabolism controlling lipid and BA homeostasis, and its activation has been shown to decrease fatty liver disease (19, 37, 38). We report that JNK-mediated repression of PPARα causes changes in BA homeostasis which suppress cholangiocyte proliferation. Consequently, JNK deficiency stimulates cholangiocyte proliferation and promotes the development of cholangiocarcinoma. This increased proliferation is mediated by the altered BA metabolism and the elevated hepatic expression of FXR/FGF15/FGFR4 that

**Fig. 1.** Hepatic JNK deficiency alters bile acid production and causes cholestasis. (A) LWT and LDKO mice (age 6 mo) were fasted overnight, and blood was collected for measuring bile acids (mean ± SEM; n = 6–11). Student’s t test differences between LDKO and LWT are indicated (**P < 0.01). (B) The composition of bile fluid collected from the gall bladder was examined by measurement of the ratio of BAs to cholesterol (Chol) or PC and the different type of BA. TCA, taurocholic acid; TMCA, taumuricholic acids. The data presented are the mean ± SEM (n = 4–5). Student’s t test differences between LDKO and LWT are indicated (*P < 0.05; **P < 0.01). (C) LKO and LWT mice (age 6 mo) were fasted overnight prior to removal of the liver. The expression of genes related to cholesterol synthesis (Hmgcr and Hmgcs1) and BA synthesis (Cyp7a1, Cyp7b1, Cyp27a1, Baat, Cyp27a1, Cyp8b1) was measured by quantitative RT-PCR (mean ± SEM; n = 5–6) and normalized to the amount of 18S RNA in each sample. Student’s t test differences between LDKO and LWT are indicated (*P < 0.05; **P < 0.01). (D) Representative liver sections prepared from mice (age 10 mo) stained with hematoxylin and eosin (H&E), an antibody to PCNA, and Masson Trichrome (Trichrome) are presented. (Scale bar, 100 μm.) (E) The expression of genes related to inflammation was evaluated by RT-PCR. (mean ± SEM; n = 5–6) and normalized to the amount of 18S RNA in each sample. Student’s t test differences between LDKO and LWT are indicated (*P < 0.05; **P < 0.01). (F) Liver damage assessed from serum measurements of ALT, AST, and γ-GT. (mean ± SEM; n = 11–24). Student’s t test differences between LDKO and LWT are indicated (**P < 0.05; **P < 0.01; ***P < 0.001).
triggers ERK activation in cholangiocytes (Fig. 6). Our results have strong translational implications for obesity treatment. Activation of FXR by BA triggers the secretion of FGF15/FGF19 in humans (39), and the beneficial effects of FGF expression on the obese metabolic profile has been well characterized (40). However, the clinical use of FGF has been debated due to the potential for increasing liver tumor development (41, 42).

Hepatic PPARα is an important mediator of this regulatory cascade. Indeed, PPARα deficiency dramatically suppresses the phenotypes caused by JNK deficiency. Nevertheless, the role of PPARα in liver cancer remains unclear. While some studies have demonstrated that PPARα activation might promote liver cancer (43–45), others indicate that PPARα activation may be neutral or suppress liver cancer development (46–49). This could be due to different experimental conditions used in these studies. In our

Fig. 2. Hepatic JNK deficiency progress to cholangiocarcinoma. (A) Representative livers of LWT and LDKO mice at age 14 mo are shown. (B) Representative sections of the liver of chow-fed LDKO mice stained with H&E and Masson Trichrome (Trichrome). (Scale bar, 100 μm.) (C) The liver mass and liver damage measured by levels of ALT and AST (mean ± SEM; n = 11–20) are presented. Student’s t test differences between LDKO and LWT are indicated (**P < 0.01, ***P < 0.001). (D) Representative liver sections of 10-mo-old LDKO and LWT mice stained with GS, Cytokeratin 19 (CK19), and Sox9. (Scale bar, 100 μm.)

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Fig. 3. ERK pathway activation accompanies the development of cholangiocarcinoma induced by hepatic JNK deficiency. (A) The expression of genes related to cholangiocytes proliferation was evaluated by RT-PCR in 14-mo-old LWT and LDKO mice and normalized to the amount of 18S RNA in each sample (mean ± SEM; n = 5–6). Student’s t test differences between LDKO and LWT are indicated (**P < 0.05; ***P < 0.01). (B) The expression of genes related to the nuclear factor FXR (Fxr, Fxrβ, Shp, Fgfr4) and Fgf15 was measured by quantitative RT-PCR in LWT and LDKO liver from mice at age 6 mo (mean ± SEM; n = 5–6) normalized to the amount of Actb mRNA in each sample. Student’s t tests between LDKO and LWT are indicated (**P < 0.05; ***P < 0.01, ***P < 0.001). (C) Representative liver sections of LDKO and LWT mice (age 10 mo) stained with phospho-ERK. (Scale bar, 100 μm.) (D) Comparative protein expression of ErbB2, phospho-ERK, and JNK1 in hepatocytes and cholangiocytes in LWT and LDKO mice was evaluated by immunoblot analysis. Vinculin was used as a loading control.
system, the protumorigenic effect of PPARα activation is due, in part, to an alteration in BA metabolism that drives ERK activation, suggesting that PPARα activation is a critical factor in cholangiocarcinoma development and progression.

The role of JNK/PPARα/FGF signaling in lipid metabolism suggests that this pathway could represent a target for the treatment for steatosis and obesity. However, the possible contribution of this pathway to carcinogenic progression represents a serious problem for long-term treatment. Our analysis suggests that treatment strategies using long-term JNK inhibition should consider the potential risk of cholangiocarcinoma development among the possible secondary effects of this treatment.

Methods

Animals. PPARα knockout mice (B6;129S4-Pparatm1Gonz/J; RRID:IMSR_JAX:008154) and Albumin-Cre mice (B6.Cg-Spre6-ps1Tg(Alb-cre)21Mng/J; RRID:IMSR_JAX:003574) were purchased from the Jackson Laboratory and backcrossed for 10 generations to the C57BL/6J background (Jackson Laboratory; RRID:IMSR_JAX:000664). Mice with compound JNK1/2 deficiency in hepatocytes (LDKO) have been described (50, 51). Genotypes were identified by PCR analysis of genomic DNA isolated from mouse tails. All experiments were performed using male mice. Mice were housed in a pathogen-free animal facility and kept on a 12-h light/dark cycle at constant temperature and humidity.


Serum Analysis. Plasma transaminase activity was assessed with the ALT and AST Reagent Kit (Biosystems Reagents) using a Benchmark Plus microplate spectrophotometer (Bio-Rad). Plasma concentration of nonsulfated bile acids was measured with the Bile Acid Assay Kit (Sigma-Aldrich) using a Fluoroskan Ascent fluorescence multiwell plate reader (Thermo Labsystems).

Table 1. PPARα deficiency reduces carcinogenesis markers in JNK1/2-deficient livers

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<th>LWT</th>
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Histochemistry. Histology was performed using tissue fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Sections (7 μm) were cut and stained using hematoxylin and eosin (American Master Tech Scientific). Sections were also incubated with Bouin’s fluid overnight, counterstain with hematoxylin (Sigma), and then stained with Masson-Trichrome stain (American Master Tech Scientific). Immunohistochemistry was performed by staining tissue sections with antibodies against PCNA (biotinylated from Thermofisher MS-106-B; RRID:AB_64272), SOX9 (Abcam ab3697; RRID:AB_304012), glutamine synthetase (Abcam ab73593; RRID:AB_2247588), cytokeratin 19 (Abcam ab15463; RRID:AB_2281021), or phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology #9101). Streptavidin-conjugated horseradish peroxidase (Biogenex) and the substrate 3,3′-diaminobenzidine (Vector Laboratories) were used followed by brief counterstaining with Mayer’s hematoxylin (Sigma).

Analysis of Biliary Lipids. Bile was collected from the gall bladder following cholecystectomy. We determined cholesterol and phospholipids using an enzymatic assay (Wako). Total bile acids were measured using Hall’s Bile Stain Kit (American MasterTech). Bile acid species were examined by a modification of the method described by ref. 52 using an HPLC-MS/MS (6410 Triple Quad LC/MS, Agilent Technologies). Chromatographic separation was achieved with gradient elution using a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm) kept at 35 °C and a flow rate of 500 μL/min. Initial mobile phase was 80:20 methanol/water, both containing 5 mM ammonium acetate and 0.01% formic acid, pH 4.6, and it was changed to 97:3 methanol/water over 9 min and then returned to 80:20 in 1 min. Electrospray ionization in negative mode was used, with the following conditions: gas temperature 350 °C, gas flow 8 L/min, nebulizer 10 psi, capillary voltage 2,500 V. MS/MS acquisition was performed in multiple reaction monitoring mode using the specific m/z transitions: [M-H]− ions to 80.2 for taurine-conjugated bile acids and [M-H]− ion to 74 for glycine-conjugated bile acids. Free bile acids did not generate characteristic ion fragments, as reported by others (52), and transition from unfragmented precursor molecular ions 407.1–407.1, 391.3–391.3, and 375.3–375.3 were selected for trihydroxylated, dehydroxylated, and monohydroxylated free bile acids, respectively.

Isolation of Hepatocytes and Cholangiocytes. Mice livers were perfused using a peristaltic pump with 40 mL of Hanks Balanced Salt Solution (with 10 mM Hepes [pH 7.4] and 1 mM EGTA; without MgCl2 and CaCl2) and then with
1 mg/mL of collagenase type I (Worthington). Hepatocytes were collected as a cell suspension in Dulbecco’s Modified Eagle’s Medium/F12 Medium (10% FBS, 0.2 mg/mL BSA, 5% sodium pyruvate, 10 mM Hepes [pH 7.4], 1% l-glutamine, 1% Penicillin/Streptomycin, 0.51 mg/mL NaHCO3). Biliary cells were collected in Williams’ medium supplemented with 5 mM Hepes (pH 7.4) and 1 mg/mL collagenase type I prior to incubation (1 h) at 37 °C to isolate cholangiocytes. The cells were washed with PBS before protein extraction as previously described (53).

Western Blot Analysis. Hepatocytes and cholangiocytes proteins were extracted in lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA pH 8.0, 50 mM sodium fluoride, 1 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.27 M sucrose, 1% Triton X-100, 0.1% SDS, 0.1% 2-mercaptoethanol, 1 μg/mL leupeptin, and 1 μg/mL aprotinin). Extracts were separated by SDS-PAGE and transferred to 0.2-μm pore-size nitrocellulose membranes (Bio-Rad). Blots were probed with primary antibodies to ErbB2 (Cat# ab16901, Abcam; RRID: AB_443537), phospho-ERK (Cat#9101, Cell Signaling Biotechnology; RRID: AB_330744), JNK1 (Cat# sc-1648, Santa Cruz Biotechnology; RRID: AB_657868), FGF-15 (Cat# sc-514647, Santa Cruz Biotechnology), GFG-21 (Cat# RD281108100, Bio-Vendor Laboratory Medicine; RRID: AB_443537), and Vinculin (Cat# V4505, Sigma; RRID: AB_477617). All antibodies were used at 1:1,000 dilution. The membranes were washed and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare), and immune complexes were detected using an enhanced chemiluminescent substrate (Clarity Western ECL substrate; Bio-Rad).

RNA-seq Analysis. Liver RNA-seq data from chow-fed LWT and LDKO (GEO GSE55190) were examined (7). Sequencing reads were preprocessed by means of a pipeline that used FastQC, to assess read quality, and Cutadapt to trim sequencing reads, eliminating Illumina adaptor remains, and to discard reads that were shorter than 30 bp. Resulting reads were mapped against reference transcriptome GRCm38.91 and quantified using RSEM. Percentages of reads participating in at least one reported alignment were around 80%. Expected expression counts calculated with RSEM were then processed with an analysis pipeline that used the Bioconductor package Limma for normalization (using TMM method) and differential expression testing, taking only into account those genes expressed with at least 1 count per million in a number of samples equal to the number of replicate samples of the condition with less replicates. Significant expression changes between wild type (WT) and JNK1/2 double-KO conditions, with Benjamini and Hochberg adjusted P value < 0.05, were detected for 44 genes. Given that the collection of differentially expressed genes was relatively small, an adjusted P value threshold of 0.2 was applied for further analyses. The resulting collection of 739 genes was then used for functional enrichment analyses with IPA (Ingenuity Pathway Analysis), to discover overrepresented gene lists derived from Ingenuity’s proprietary knowledge-base (IPA KB). IPA KB-derived gene lists consisted of collections of genes belonging to the same signaling or metabolic pathway (Canonical Pathway analyses), or regulated by the same molecule (Upstream Regulator analyses). In general, enrichments associated to Benjamini-Hochberg adjusted P value < 0.05 are considered significant. Importantly, IPA may issue predictions on the activation state of pathways or regulators in the form of a parameter called z score; activation or inhibition is indicated by positive or negative values, respectively. Other data manipulations and graphical representations (heatmaps, bar plots, and scatter plots) were produced with statistical package R.

Real-Time qPCR. Total RNA was isolated from liver and tumor tissue using the RNeasy Mini Kit (Qiagen) with on-column DNase I-digestion. cDNA (complementary DNA) was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Taqman® assays were performed using the probes listed in SI Appendix, Table S1 (Applied Biosystems). Sequences of primers used for quantitative real-time PCR (RT-PCR) are provided in SI Appendix, Table S2. Expression levels were normalized to 18S (Figs. 3, 4, and 5) mRNA. qRT-PCR was performed using the Fast SYBR Green system (Applied Biosystems) in a 7900HT Fast Real-Time PCR thermal cycler (Applied Biosystems). A dissociation curve program was employed after each reaction to verify purity of the PCR products. The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast Real-Time PCR machine.

Statistics. Differences between groups were examined for statistical significance using two-tailed unpaired Student’s t test (with Welch’s correction when variances were different) or ANOVA coupled to Bonferroni’s post-test. Kaplan-Meier analysis was performed using the log-rank test. Statistical details and experimental n are specified in figure legends.

Fig. 6. Schematic illustration of JNK-regulated cholangiocarcinoma development in the liver.


7. S. Vernia et al., The PPARα-FG31 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. Cell Metab. 20, 512–525 (2019).


