Fibroblast Growth Factor 21 Mediates Glycemic Regulation by Hepatic JNK

Graphical Abstract

Highlights
- Hepatic JNK activity regulates FGF21 expression during fasting/feeding cycles
- Hepatic JNK suppresses circulating FGF21 and promotes metabolic syndrome
- Hepatocyte FGF21 is required for the effects of hepatic JNK on metabolic syndrome

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In Brief
Vernia et al. examine the role of circulating FGF21 in the response to JNK signaling. Hepatic JNK deficiency promotes expression of FGF21 and improves glycemia. Liver-specific ablation of the Fgf21 gene prevents this improvement of glycemia. These data argue that FGF21 mediates metabolic actions of hepatic JNK.
Fibroblast Growth Factor 21 Mediates Glycemic Regulation by Hepatic JNK

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SUMMARY

The cJun NH2-terminal kinase (JNK)-signaling pathway is implicated in metabolic syndrome, including dysregulated blood glucose concentration and insulin resistance. Fibroblast growth factor 21 (FGF21) is a target of the hepatic JNK-signaling pathway and may contribute to the regulation of glycemia. To test the role of FGF21, we established mice with selective ablation of the Fgf21 gene in hepatocytes. FGF21 deficiency in the liver caused marked loss of FGF21 protein circulating in the blood. Moreover, the protective effects of hepatic JNK deficiency to suppress metabolic syndrome in high-fat diet-fed mice were not observed in mice with hepatocyte-specific FGF21 deficiency, including reduced blood glucose concentration and reduced intolerance to glucose and insulin. Furthermore, we show that JNK contributes to the regulation of hepatic FGF21 expression during fasting/feeding cycles. These data demonstrate that the hepatokine FGF21 is a key mediator of JNK-regulated metabolic syndrome.

INTRODUCTION

The cJun NH2-terminal kinase (JNK)-signaling pathway is activated by metabolic stress and contributes to the development of metabolic syndrome in response to the consumption of a high-fat diet (HFD), including obesity, hyperglycemia, and insulin resistance (Sabio and Davis, 2010). JNK plays different roles in multiple tissues to cause these phenotypes. Thus, JNK in the hypothalamus and pituitary gland suppress energy expenditure to cause obesity (Belgardt et al., 2010; Sabio et al., 2010a; Vernia et al., 2013), and JNK in macrophages promotes chronic inflammation to cause insulin resistance (Han et al., 2013). JNK in peripheral tissues also contributes to the development of insulin resistance, including adipose tissue, liver, and muscle (Sabio et al., 2008, 2010b; Sabio and Davis, 2010). Interestingly, hepatic JNK deficiency causes systemic protection against insulin resistance in HFD-fed mice (Vernia et al., 2014). The mechanism of systemic protection may be caused by JNK-mediated repression of the peroxisome proliferator-activated receptor α (PPARα)/fibroblast growth factor 21 (FGF21)-signaling axis (Vernia et al., 2014).

FGF21 circulates in the blood and is a potent regulator of metabolism (Owen et al., 2015). The effects of FGF21 are mediated by binding to FGF receptors (FGFR1c, 2c, or 3c) together with the obligate co-receptor βKlotho (Owen et al., 2015). Clinical trials using FGF21 analogs demonstrate improved dyslipidemia in humans (Gaich et al., 2013; Dong et al., 2015). Murine studies demonstrate that the liver is a target of the metabolic actions of FGF21 by regulating fatty acid oxidation, ketogenesis, gluconeogenesis, and lipogenesis (Potthoff et al., 2012). Indeed, FGF21 deficiency is associated with increased hepatic steatosis, fibrosis, and inflammation (Fisher et al., 2014; Tanaka et al., 2013). Direct effects of FGF21 on the liver may contribute to these hepatic responses, but recent studies indicate that FGF21 primarily mediates these effects by indirect mechanisms that initially target other tissues, including the CNS and adipose tissue (Owen et al., 2015). The major hepatic action of FGF21 mediated by adipose tissue is the PPARγ-induced expression of the adipokine adiponectin (Holland et al., 2013; Lin et al., 2013). FGF21 also acts on the hypothalamus-pituitary axis to regulate adrenal glucocorticoid secretion during starvation-induced hepatic gluconeogenesis (Liang et al., 2014). Moreover, FGF21 acts on the hypothalamus within the suprachiasmatic nucleus (Owen et al., 2014) and the paraventricular nucleus (Douris et al., 2015) to increase sympathetic outflow to peripheral tissues, including the liver. This sympathetic activity also targets brown fat and beige/brite cells in subcutaneous white fat depots (Douris et al., 2015), and it may contribute to the blood glucose-lowering actions of FGF21 by causing increased UCP1-dependent energy expenditure (Kwon et al., 2015).

The purpose of the study reported here was to test the role of FGF21 in the metabolic response to hepatic JNK activation. We show that hepatic JNK deficiency in HFD-fed mice causes reduced hyperglycemia and improved tolerance to glucose and insulin. These effects of JNK deficiency were not detected in mice with hepatocyte-specific ablation of the Fgf21 gene. Collectively, these data demonstrate that FGF21 is required for glycemic regulation by hepatic JNK.
RESULTS AND DISCUSSION

JNK Signaling Suppresses Fgf21 Gene Expression

Studies of mice with compound ablation of the Mapk8 plus Mapk9 genes (also known as Jnk1 and Jnk2) in the liver demonstrate increased expression of PPARα target genes, including Fgf21 (Vernia et al., 2014). The JNK-signaling pathway can therefore repress PPARα signaling. To obtain additional evidence to support this conclusion, we examined the effect of a specific small molecule inhibitor of JNK protein kinase activity (JNK-in-8; Zhang et al., 2012) on PPARα-dependent gene expression, including Acox1, Ehhadh, and Fgf21. We found that treatment of wild-type hepatocytes, but not Ppara−/− hepatocytes, with JNK-in-8 caused increased PPARα-dependent gene expression in response to the PPARα agonist WY14043 (Figure 1A). These data support the conclusion that JNK signaling represses PPARα target gene expression, including Fgf21. This PPARα pathway is important for starvation-induced FGF21 expression (Badman et al., 2007; Inagaki et al., 2007), while additional pathways (e.g., ATF4, ChREBP, GCN2, NRF2, and SIRT1) play key roles in FGF21 expression caused by other dietary stresses, including low-protein and high-carbohydrate diets (Iizuka et al., 2009; Chartoumpekis et al., 2011; De Sousa-Coelho et al., 2012; Li et al., 2014).

To obtain independent evidence for repression of Fgf21 expression by the JNK-signaling pathway, we examined the effect of defects in the upstream regulatory mechanism that activates JNK. It is established that JNK activation is mediated by the protein kinases MKK4 and MKK7 (Tournier et al., 2001). Disruption of the Map2k4 gene or the Map2k7 gene in hepatocytes partially suppresses JNK signaling, indicated by reduced phosphorylation of JNK and cJun (Figures 1B and 1C). In contrast, compound ablation of Map2k4 plus Map2k7 (like compound ablation of Mapk8 plus Mapk9) prevented JNK signaling in hepatocytes (Figure 1C). Analysis of Fgf21 mRNA expression demonstrated that both MKK4 deficiency and MKK7 deficiency caused increased Fgf21 expression, but compound MKK4 plus MKK7 deficiency strongly increased Fgf21 expression (Figure 1E).

Similarly, compound deficiency of the mixed-lineage protein kinases MLK2 plus MLK3, which function as activators of MKK4 and MKK7 in response to metabolic stress (Kant et al., 2013), causes increased expression of FGF21 circulating in the blood (Figure 1D). Together, these data demonstrate that the JNK-signaling pathway acts to suppress FGF21 expression.

To test the role of JNK signaling during physiological regulation of FGF21 expression, we investigated the effect of fasting and re-feeding mice on hepatic FGF21 expression and JNK activity. It is established that FGF21 expression is strongly induced by starvation and that re-feeding rapidly represses FGF21 expression when...
Conditional Ablation of the Fgf21 Gene in Mice

We established Fgf21<sup>Cre<sub>LoxP<sub>LoxP</sub></sup> mice to examine the effect of tissue-specific ablation of the Fgf21 gene (Figures S1A and S1B). To test the role of hepatic FGF21 expression, we obtained L<sup>ΔFGF21</sup> mice (Ab<sup>-</sup>-<sup>c</sup> Fgf21<sup>Cre<sub>LoxP<sub>LoxP</sub></sup></sup>) with Fgf21 gene ablation selectively in hepatocytes. Analysis of L<sup>ΔFGF21</sup> mice demonstrated no hepatic expression of Fgf21 mRNA or FGF21 protein circulating in blood (Figures S1C–S1E). In contrast, F<sup>ΔFGF21</sup> mice (Adipoq<sup>-</sup>-<sup>c</sup> Fgf21<sup>Cre<sub>LoxP<sub>LoxP</sub></sup></sup>) mice with Fgf21 gene ablation selectively in adipose tissue demonstrated the presence of FGF21 protein in blood, despite the complete loss of Fgf21 mRNA expression in adipose tissue (Figures S1C–S1E). These observations confirm that the liver is the major source of FGF21 circulating in blood (Markan et al., 2014).

Hepatic FGF21 Deficiency Promotes Metabolic Syndrome

We examined the effect of feeding a HFD to control mice and mice with FGF21 deficiency in liver (L<sup>ΔFGF21</sup>) or adipose tissue (F<sup>ΔFGF21</sup>). Measurement of body mass demonstrated increased weight gain by L<sup>ΔFGF21</sup> mice, but not F<sup>ΔFGF21</sup> mice, compared with control mice (Figures 3A and S2A). Analysis of total body composition by H-MRS demonstrated that the increased body mass was caused by accumulated fat mass rather than increased lean mass (Figure 3B). Examination of organ mass at necropsy indicated that the majority of the increased weight gain was associated with increased mass of the liver, white adipose tissue, and brown adipose tissue (Figure S2B). Sections prepared from the liver demonstrated that L<sup>ΔFGF21</sup> mice exhibited increased hepatic steatosis compared with F<sup>ΔFGF21</sup> mice and control mice (Figure 3D). Similarly, analysis of sections prepared from brown and white adipose tissues demonstrated increased adipocyte hypertrophy in L<sup>ΔFGF21</sup> mice compared with F<sup>ΔFGF21</sup> mice and control mice (Figures 3E and 3F). The increased obesity of L<sup>ΔFGF21</sup> mice was associated with increased HFD-induced hyperinsulinemia and circulating concentrations of the adipokines leptin and resistin (Figures S2C–S2E). This effect of hepatic FGF21 deficiency to promote obesity is similar to observations reported for whole-body FGF21 knockout mice (Badman et al., 2009; Hotta et al., 2009).

The phenotype of L<sup>ΔFGF21</sup> mice suggests that hepatic FGF21 deficiency may promote metabolic syndrome. We therefore examined glycemic regulation in L<sup>ΔFGF21</sup> mice, F<sup>ΔFGF21</sup> mice, and control mice. No significant differences in blood glucose concentration were detected in CD-fed mice. Similarly, studies of glucose, insulin, and pyruvate tolerance of CD-fed mice demonstrated no differences among control mice, L<sup>ΔFGF21</sup> mice, and F<sup>ΔFGF21</sup> mice (Figures S3A–S3C). In contrast, the blood glucose concentration was increased in HFD-fed L<sup>ΔFGF21</sup> mice compared with HFD-fed F<sup>ΔFGF21</sup> mice and HFD-fed control mice (Figure 3C). Furthermore, HFD-fed L<sup>ΔFGF21</sup> mice were significantly more glucose intolerant (Figure 3G), more insulin resistant (Figure 3H), and more pyruvate intolerant (Figure 3I) than HFD-fed control mice or F<sup>ΔFGF21</sup> mice.

Collectively, these data demonstrate that hepatocyte-specific FGF21 deficiency dramatically suppresses the amount of FGF21 that circulates in the blood. Moreover, hepatocyte-specific expression (Potthoff et al., 2012). Previous studies have demonstrated that hepatic JNK is activated by over-nutrition when mice are fed a HFD (Sabio and Davis, 2010). However, it was not known whether hepatic JNK is acutely regulated by fasting and re-feeding. We therefore examined JNK activation in mice fed a chow diet (CD) ad libitum, fasted overnight, or fasted overnight and then re-fed a CD (1 hr). Liver extracts were prepared and examined by immunoblot analysis (left). The amounts of phospho-JNK and phospho-cJun (right) were quantitated (mean ± SEM; n = 6–10). The blood concentration of FGF21 (C) was measured by ELISA (mean ± SEM; n = 10–12). Statistically significant differences between L<sup>WT</sup> and L<sup>L<sub>J1,J2</sub></sup> mice are indicated (**p < 0.01).

Figure 2. Hepatic JNK Regulates FGF21 Levels in Response to Fasting

(A) Mice were fed a CD ad libitum, fasted overnight, or fasted overnight and then re-fed a CD (1 hr). Liver extracts were prepared and examined by immunoblot analysis (left). The amounts of phospho-JNK and phospho-cJun (right) were quantitated (mean ± SEM; n = 3; *p < 0.05 and **p < 0.01). (B and C) The amount of hepatic Fgf21 mRNA (B) was measured by qPCR (mean ± SEM; n = 6–10). The blood concentration of FGF21 (C) was measured by ELISA (mean ± SEM; n = 10–12). Statistically significant differences between L<sup>WT</sup> and L<sup>L<sub>J1,J2</sub></sup> mice are indicated (**p < 0.01).
FGF21 deficiency promotes obesity and hallmarks of metabolic syndrome in HFD-fed mice, including increased hyperglycemia and hyperinsulinemia together with intolerance to glucose, pyruvate, and insulin.

**FGf21 Gene Ablation Suppresses the Metabolic Effects of Hepatic JNK Deficiency**

Hepatic JNK deficiency causes increased expression of PPARα target genes in the liver, including *Fgf21* (Vernia et al., 2014). To test the role of FGF21, we examined the effect of compound deficiency of JNK plus FGF21 in hepatocytes by comparing control mice (Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/LoxP) with hepatic JNK-deficient mice (Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP) and adipose tissue-specific FGF21-deficient mice (Lβt2cre+ Mapk9LoxP/LoxP Fgf21LoxP/−).

No significant differences in total body mass, fat mass, or lean mass among Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/LoxP mice, and control mice were detected (Figures 4A and 4B). However, the HFD-induced hyperglycemia in control mice that was suppressed by JNK deficiency (compare control and Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/LoxP mice; Figure 4C) was not detected in FGF21-deficient mice (compare Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/LoxP and Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/− mice; Figure 4C). These data indicate that FGF21 is essential for the effect of hepatic JNK deficiency to suppress HFD-induced hyperglycemia.

To examine the requirement of FGF21 for glycemic regulation, we performed glucose, insulin, and pyruvate tolerance tests. Studies of CD-fed mice demonstrated no significant differences between groups (Figures S3D–S3F), but JNK deficiency in HFD-fed mice caused improved performance in each of these tests (Figure 4D). This effect of JNK deficiency was not detected in FGF21-deficient mice (compare HFD-fed Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/− and HFD-fed Lβt2cre+ Mapk8LoxP/LoxP Mapk9LoxP/LoxP Fgf21LoxP/LoxP Fgf21LoxP/− mice; Figure 4D). Moreover, hepatic JNK deficiency did not suppress HFD-induced hyperinsulinemia or circulating concentrations of the adipokines leptin and resistin in liver-specific FGF21-deficient mice (Figure 4E). Together, these data confirm that FGF21 is required for improved glycemic regulation caused by hepatic JNK deficiency.

Interestingly, the effect of hepatic JNK deficiency to increase the expression of the PPARα target genes Acox1 and Ehhadh in HFD-fed mice was suppressed in hepatic FGF21-deficient mice (Figure 4F). This observation suggests that FGF21 plays a key role in the promotion of fatty acid oxidation caused by hepatic JNK deficiency, consistent with the established physiological
Figure 4. FGF21 Is Critically Required for the Metabolic Actions of Hepatic JNK

(A) The total body mass gain of CD-fed and HFD-fed mice was examined. The effect of FGF21 deficiency in liver was examined by comparing control mice (Mapk8LoxP/LoxP, Mapk9LoxP/LoxP) and mice with liver-specific deficiency of JNK (L^L^D^J1,J2) or JNK plus FGF21 (L^L^D^J1,J2,Fgf21). No statistically significant differences were detected between groups (mean ± SEM; n = 8–10).

(B) The fat and lean mass of CD-fed and HFD-fed (12 weeks) mice were measured by 1H-MRS analysis (mean ± SEM; n = 8–10).

(C) Blood glucose concentration was measured in overnight-fasted CD-fed and HFD-fed (12 weeks) mice (mean ± SEM; n = 6–10; *p < 0.05 and ***p < 0.001).

(D) GTT, ITT, and PTT using HFD-fed (12 weeks) mice were performed (mean ± SEM; n = 6–10; *p < 0.05, **p < 0.01, and ***p < 0.001).

(E) The blood concentrations of insulin, leptin, and resistin in overnight-fasted CD-fed and HFD-fed (12 weeks) mice were measured (mean ± SEM; n = 6–10; *p < 0.05, **p < 0.01, and ***p < 0.001).

(F) The expression of Acox1, Ehhadh, and Fgf21 mRNA in the liver of CD-fed and HFD-fed (12 weeks) mice were measured by qPCR (mean ± SEM; n = 6; *p < 0.05, **p < 0.01, and ***p < 0.001).

(G) Sections of liver from CD-fed and HFD-fed (12 weeks) control, L^L^D^J1,J2, and L^L^D^J1,J2,Fgf21 mice were stained with H&E. Scale bar, 100 μm.

See also Figures S3 and S4.
role of FGF21 (Badman et al., 2007). Indeed, the reduction in HFD-induced hepatic steatosis caused by JNK deficiency was not detected in the liver of mice with hepatic FGF21 deficiency (Figure 4G). These data highlight the crucial role of FGF21 in hepatic JNK signal transduction.

One pathway that mediates the hepatic actions of FGF21 is represented by the adipokine adiponectin (Holland et al., 2013; Lin et al., 2013). We therefore examined the expression of adiponectin in control, L6,J1,J2, and F6,J1,J2,Fgf21 mice; these data demonstrated that hepatic FGF21 deficiency, but not adipocty FGF21 deficiency, caused reduced expression of Adipoq mRNA in epididymal white adipose tissue (Figure S4A). Moreover, studies of control, L6,J1,J2, and L6,J1,J2,Fgf21 mice indicated that hepatic JNK deficiency caused increased adiponectin expression that was suppressed in mice with compound hepatic deficiency of JNK plus FGF21 (Figure S4B). Together, these data demonstrate that the increased circulating concentration of FGF21 caused by hepatic JNK deficiency promotes adipose tissue expression of adiponectin. The FGF21-promoted adiponectin expression, together with FGF21-promoted sympathetic stimulation of brown and beige/brite adipose tissue, may contribute to the improved hepatic function of mice with hepatic JNK deficiency.

In summary, the requirement of FGF21 for the metabolic actions of hepatic JNK provides a mechanism for the hepatic metabolic regulation by the JNK-signaling pathway in the liver. FGF21 plays a major role in the response to starvation, including increased hepatic gluconeogenesis, ketogenesis, and fatty acid oxidation (Pothoff et al., 2012). Feeding terminates this response by activation of the hepatic JNK-signaling pathway and repression of FGF21 expression. JNK activation, therefore, provides a regulatory mechanism that contributes to the termination of the starvation response. Conversely, starvation-induced inhibition of the JNK pathway contributes to de-repression of FGF21 expression and promotes adaptive responses to starvation. These data establish a key role played by FGF21 in the metabolic response to hepatic JNK activation caused by cycles of fasting and re-feeding.

The interplay between hepatic JNK and FGF21 contributes to the development of metabolic syndrome in response to diet-induced obesity (Vernia et al., 2014). Indeed, the protective effects of hepatic JNK deficiency on insulin resistance were not observed in mice with hepatic FGF21 deficiency (Figure 4). This finding is similar to a report of autophagy-deficient mice that exhibit improved metabolic syndrome that also was dependent on increased FGF21 expression (Kim et al., 2013). Similarly, FGF21 improves hyperglycemia in mice with hepatic deficiency of the insulin receptor (Emanuelli et al., 2014). FGF21 also can provide protection against some forms of drug-induced hepatotoxicity (Ye et al., 2014). Together, these data demonstrate that FGF21 can play strong protective roles in models of metabolic and cellular dysfunction.

EXPERIMENTAL PROCEDURES

Blood Analysis

Blood glucose was measured with an Ascensia Breeze 2 glucometer (Bayer). FGF21 was measured using the Rat/Mouse Fibroblast Growth Factor 21 ELISA kit (EZR-MFGF21-26K, Millipore). Adipokines and insulin in plasma were measured by multiplexed ELISA using a Luminex 200 machine (Millipore).

Glucose and Insulin Tolerance Tests

Glucose, insulin, and pyruvate tolerance tests were performed by intraperitoneal injection of mice with glucose (1 g/kg), insulin (0.5 U/kg), or pyruvate (1 g/kg) using methods described previously (Sabio et al., 2008).

RNA Analysis

Tissue isolated from mice starved overnight was used to prepare total RNA with the RNeasy mini kit (Qiagen). Total RNA (500 ng) was converted into cDNA using the high-capacity cDNA reverse transcription kit (Life Technologies). The diluted cDNA was used for real-time qPCR analysis using a QuantStudio PCR machine (Life Technologies). TaqMan assays (Life Technologies) were used to quantify Acox1 (Mm01246831_m1), Adipoq (Mm00456425-m1), and Ehhadh (Mm00619685_m1). Fgf21 expression was quantified using the primers FGF21F67 (5’-AGATGGAGCTCTCTATGGATCG-3’) and FGF21R67 (5’-GGGCTTCAGACTGGTACACAT-3’) with universal probe 67 (Universal Probe Library, Roche). The relative mRNA expression was normalized by measurement of the amount of 18S RNA in each sample using Taqman assays (catalog number 4308329, Life Technologies).

Immunoblot Analysis

Liver extracts were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 μM sodium orthovanadate, 1 μM PMSF, and 10 μg/ml leupeptin plus aprothin). Experiments (30–50 μg protein) were examined by immunoblot analysis by probing with antibodies to pSer63-cJUN, pJNK, pMKK4, MKK4, pMKK7, and MKK7 (Cell Signaling Technology); JNK1/2 (BD Pharmingen); cJUN and GAPDH (Santa Cruz Biotechnology); and β-Tubulin (Covance). Immune complexes were detected by fluorescence using anti-mouse and anti-rabbit secondary IRDye antibodies (LI-COR Biosciences) and quantitated using the LI-COR Imaging system.

Analysis of Tissue Sections

Histology was performed using tissue fixed in 10% formalin (24 hr), dehydrated, and embedded in paraffin. Sections (7 μm) were cut and stained using H&E (American Master Tech Scientific). Sections isolated from five mice per group were examined by a board-certified pathologist who was blinded with respect to the identity of each group of mice.

Statistical Analysis

Differences between groups were examined for statistical significance using the Student’s test or ANOVA with the Fisher’s test.

Institutional Review Board Approval

The Institutional Animal Care and Use Committee of the University of Massachusetts Medical School approved all studies using animals.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.026.

AUTHOR CONTRIBUTIONS

S.V. and R.J.D. designed the study. S.V., J.C.-K., and T.B. performed experiments. S.V., C.T., and R.J.D. analyzed data and wrote the paper.

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