Brief Communication

MOLECULAR METABOLISM

Induction of fibroblast growth factor 21 does not require activation of the hepatic X-box binding protein 1 in mice

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ABSTRACT

Objective: Fibroblast growth factor 21 (FGF21), a key regulator of the metabolic response to fasting, is highly induced by endoplasmic reticulum (ER) stress. The X-box binding protein 1 (*Xbp1*) is one of several ER stress proteins that has been shown to directly activate the FGF21 promoter. We aimed to determine whether hepatic *Xbp1* is required for induction of hepatic FGF21 *in vivo*.

Methods: Mice bearing a hepatocyte-specific deletion of *Xbp1* ($Xbp1^{LKO}$) were subjected to fasting, pharmacologic ER stress, or a ketogenic diet, all potent stimuli of *Fgf21* expression.

Results: Hepatocyte-specific *Xbp1* knockout mice demonstrated normal induction of FGF21 in response to fasting or pharmacologic ER stress and enhanced induction of FGF21 in response to a ketogenic diet. Consistent with preserved induction of FGF21, $Xbp1^{LKO}$ mice exhibited normal induction of FGF21 target genes and normal ketogenesis in response to fasting or a ketogenic diet.

Conclusion: Hepatic *Xbp1* is not required for induction of FGF21 under physiologic or pathophysiologic conditions *in vivo*.

Keywords Unfolded protein response; Endoplasmic reticulum stress; Fasting; Fatty acid oxidation; Ketogenic diet

1. INTRODUCTION

Fasting induces well-characterized changes in hepatic lipid and glucose metabolism that are critical to maintaining energy balance within an organism. Fibroblast growth factor 21 (FGF21) has emerged as a key regulator of the metabolic response to fasting [1–3]. In the fasted state, increased serum free fatty acids released from lipolysis of adipose tissue lead to activation of peroxisome proliferator-activated receptor α (PPARα), a major inducer of FGF21 [1,2,4]. FGF21, in turn, promotes fatty acid β-oxidation, gluconeogenesis, and ketogenesis, key adaptive responses to starvation [5,6]. In addition to its physiologic role in mediating the metabolic response to fasting, FGF21 is increasingly recognized to have broader metabolic actions. Moreover, induction of FGF21 is a well-established feature of both rodent and human metabolic diseases such as obesity, type II diabetes, and nonalcoholic fatty liver disease (NAFLD) [7–17]. Furthermore, systemically administered FGF21 has been shown to lower blood glucose and triglycerides, and improve obesity, hepatic steatosis, and insulin resistance [18–21]. Interestingly, in humans, FGF21 is not dramatically induced by short-term fasting but is robustly induced by fructose or sucrose ingestion [13,22–24]. These observations have raised speculation that the primary function of FGF21 in humans may be to counteract metabolic disease rather than mediate the adaptive response to starvation. Given the massive public health burden that obesity and its sequelae impose, the regulation of FGF21 has been an area of intense investigation in recent years.

In addition to fasting and metabolic disturbance, endoplasmic reticulum (ER) stress is now a well-established inducer of hepatic FGF21 expression [25,26]. ER stress and the ensuing unfolded protein response (UPR), have been increasingly implicated in the pathogenesis of metabolic diseases such as obesity, type II diabetes, and NAFLD. As such, it has been speculated that induction of FGF21 by ER stress may serve to counteract the negative metabolic consequences of unresolved ER stress [25,27,28].

Although it is widely accepted that ER stress, among other cellular stressors, induces hepatic FGF21 expression, the mechanism remains controversial. ER stress response elements have been identified in the *Fgf21* promoter that are capable of binding several ER stress-induced transcription factors [25–27]. The PERK-eIF2α-ATF4 branch of the UPR in particular has clearly been shown to mediate ER stress-induced FGF21 expression *in vitro* and *in vivo* [25,28]; however, the physiologic role of other ER stress-induced transcription factors in the regulation of FGF21 is less well understood.

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Abbreviations: FGF21, fibroblast growth factor 21; XBP1, X-box binding protein 1; IRE1, inositol requiring enzyme 1; UPR, unfolded protein response; ER, endoplasmic reticulum; PPARα, proliferator-activator receptor alpha; Pgc1α, PPAR-gamma cofactor 1α; Cpt1α, carnitine palmitoyl acyl-CoA transferase 1; Aco, acyl-CoA-oxidase; KD, ketogenic diet; shRNA, short hairpin RNA; CHOP, C/EBP homologous protein; Atf4, activating transcription factor 4; eIF2α, eukaryotic translation initiation factor 2-alpha; Srebp1c, sterol regulatory element binding protein 1c; Fas, fatty acid synthase; Dgat2, diacylglycerol acyltransferase 2; Scd-1, stearoyl-CoA-desaturase 1

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The IRE1α-XBP1 branch of the UPR has been strongly implicated in regulating hepatic lipid and glucose metabolism, making this particular branch of the UPR an intriguing candidate for mediating the effect of ER stress on FGF21 expression [29–31]. XBP1 has been shown to bind to the promoter of FGF21 *in vitro*, and there has been recent interest in and controversy over the role of XBP1 in the regulation of FGF21 [25–27]. *In vitro* experiments have shown that overexpression of XBP1s in primary hepatocytes induces *FGF21* expression whereas knockdown of *XBP1* blunts *FGF21* induction [27]. Furthermore, it has previously been shown that mice bearing a liver-specific deletion of *Ire1α*, the primary activator of *Xbp1*, show impaired activation of *Fgf21* when challenged with pharmacologic ER stress [27]. Despite this compelling data, the role of hepatic *Xbp1* in the regulation of hepatic *Fgf21* under physiologic or pathophysiologic conditions remains unclear. To directly determine whether hepatic *Xbp1* is required for induction of hepatic *Fgf21 in vivo*, we subjected mice bearing a hepatocyte-specific deletion of *Xbp1* to fasting, a ketogenic diet, or pharmacologic ER stress, all potent stimuli of *Fgf21* expression.

2. EXPERIMENTAL PROCEDURES

2.1. Animals and treatments

C57BL/6 -*Xbp1*$^{fl/fl}$ mice with loxP sites flanking exon 2 of the *Xbp1* gene were kindly provided by Dr. Laurie H. Glimcher. The generation of the *Xbp1*$^{fl/fl}$ strain has been previously reported [29]. *Xbp1*$^{fl/fl}$ mice were bred with C57BL/6 -Albumin-Cre mice (Jackson Laboratory, ME) that express Cre-recombinase in albumin-producing hepatocytes as previously described [32]. *Xbp1*$^{fl/fl}$ mice expressing Cre recombinase were confirmed to be liver-specific *Xbp1*-knockout mice (*Xbp1*^{LKO}) by western blot for XBP1 and real-time PCR using primers targeting a deleted region of the transcript in exon 2. Littermate *Xbp1*$^{fl/fl}$ mice negative for expression of Cre recombinase were used as control mice. To determine the effect of hepatic *Xbp1* deletion on fasting-induced FGF21 induction, female *Xbp1*^{LKO} mice and *Xbp1*$^{fl/fl}$ controls (6 weeks of age) were fasted overnight (18 h, from 4pm to 10am). To study the effects of a ketogenic diet in mice lacking hepatic *Xbp1*, female *Xbp1*^{LKO} mice and *Xbp1*$^{fl/fl}$ controls (6 weeks of age) were fed a high-fat, ketogenic diet (KD) for 2 weeks. Given the potential confounding variables of sex and age on FGF21 expression, we also fed aged (20 week old), male *Xbp1*^{LKO} mice and *Xbp1*$^{fl/fl}$ controls a KD for 2 weeks. The KD is composed of 84% fat, 16% protein, 0% carbohydrate (TestDiet, St. Louis, MO). Food intake was measured in KD-fed mice and found to be similar among *Xbp1*^{LKO} mice and *Xbp1*$^{fl/fl}$ controls. To determine the effect of pharmacologic ER stress on the regulation of FGF21, female *Xbp1*^{LKO} mice and *Xbp1*$^{fl/fl}$ controls (6 weeks of age) were treated with a single intraperitoneal injection of tunicamycin (0.5 mg/kg) or vehicle (20% DMSO/PBS) and were sacrificed 6 h post-injection. All mice underwent 14/10-hour light/dark cycling before and during the treatment protocol and were given free access to water during dietary manipulation. Mice were sacrificed by CO_2 inhalation followed by cardiac puncture. The cardiac blood was immediately centrifuged to collect the plasma. The livers were rapidly excised, flushed with ice-cold saline, and sectioned. An aliquot of liver was fixed in formalin and the remaining liver was snap-frozen in liquid nitrogen. All animal protocols were approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC).

2.2. Liver and plasma chemistries

Plasma FGF21 levels were measured using an FGF21 ELISA assay kit (RND Systems, Minneapolis, MN). Plasma β-hydroxybutyrate levels were measured using a biochemical assay (Stanbio Laboratory, Boerne, TX). Glucose was measured on whole blood obtained by tail bleed using a One Touch Ultra glucometer (Lifescan, Milpitas, CA). Plasma insulin was measured using an Insulin ELISA assay kit (Thermo Scientific, Frederick, MD). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as (fasting glucose (in mg/dL) X fasting insulin (mU/L))/405. Plasma FGF21, β-hydroxybutyrate, and insulin were measured on plasma collected from cardiac blood at the termination of the experiment. Liver samples were homogenized in Dulbecco's phosphate buffered saline for hepatic lipid analysis (100 mg liver tissue/1 mL). Triglyceride levels were measured in liver homogenate using an Infinity spectrophotometric assay per the manufacturer's protocol (Thermo Electron Corporation, Melbourne, Australia).

2.3. Cell culture experiments

Human hepatoma Huh7 cells transfected with short hairpin RNA (shRNA) targeting XBP1 (Huh7^{shXBP1}) or control shRNA (Huh7^{shCON}) were generated and characterized as previously described [33] (kindly provided by Dr. Richard Green, Northwestern University, Chicago, IL). Huh7^{shCON} and Huh7^{shXBP1} cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin at 37 °C with 5% CO2. To induce ER stress, cells were grown to 80% confluence in 6-well plates and treated with tunicamycin (5 μg/mL) or vehicle (DMSO/saline) in serum-free media for 6 h. RNA isolation was performed using TRIzol Reagent (Ambion Life Technologies, Carlsbad, CA) per protocol.

2.4. Analysis of gene and protein expression

Total RNA from frozen liver or cultured cells was isolated using TRIzol reagent, and real-time quantitative PCR was performed as previously described [34,35]. Total protein was isolated from frozen liver samples, and western blotting was performed as previously described [34,35]. Protein detection was performed using polyclonal rabbit antibodies to IRE1α, CHOP, total and phosphorylated eIF2α, and GAPDH (Cell Signaling Technology, Danvers, MA). Bound antibody was detected using goat anti-rabbit polyclonal HRP antibody (Cell Signaling Technology) and developed using ECL Western Blotting Substrate (Cell Signaling Technology). Representative western blots of pooled samples are shown.

2.5. Statistical analysis

Data are presented as mean ± standard deviation (SD). Comparisons between groups were performed using Student's *t*-test analysis.

3. RESULTS

3.1. Hepatic *Xbp1* is not required for induction of hepatic *Fgf21* in response to fasting

We first examined the effect of hepatic *Xbp1*-deletion on induction of *Fgf21* in response to fasting, a potent stimulus of hepatic *Fgf21* expression. As expected, fasting markedly increased the hepatic expression of *Fgf21* in *Xbp1*$^{fl/fl}$ control mice (Figure 1A). Paralleling the induction of hepatic *Fgf21* expression, the plasma concentration of FGF21 was also markedly increased by fasting (Figure 1B). Surprisingly, *Xbp1*^{LKO} mice showed an equivalent increase in hepatic *Fgf21* expression and plasma FGF21 concentration relative to *Xbp1*$^{fl/fl}$ mice in response to fasting (Figure 1A,B). These findings indicate that hepatic *Xbp1* does not mediate fasting-related hepatic *Fgf21* induction.

Having shown that *Xbp1*^{LKO} mice demonstrate normal induction of hepatic *Fgf21* in response to fasting, we next examined whether fasted *Xbp1*^{LKO} mice show normal induction of FGF21-mediated downstream signaling. FGF21 has been shown to induce hepatic *Pgc1α* expression;

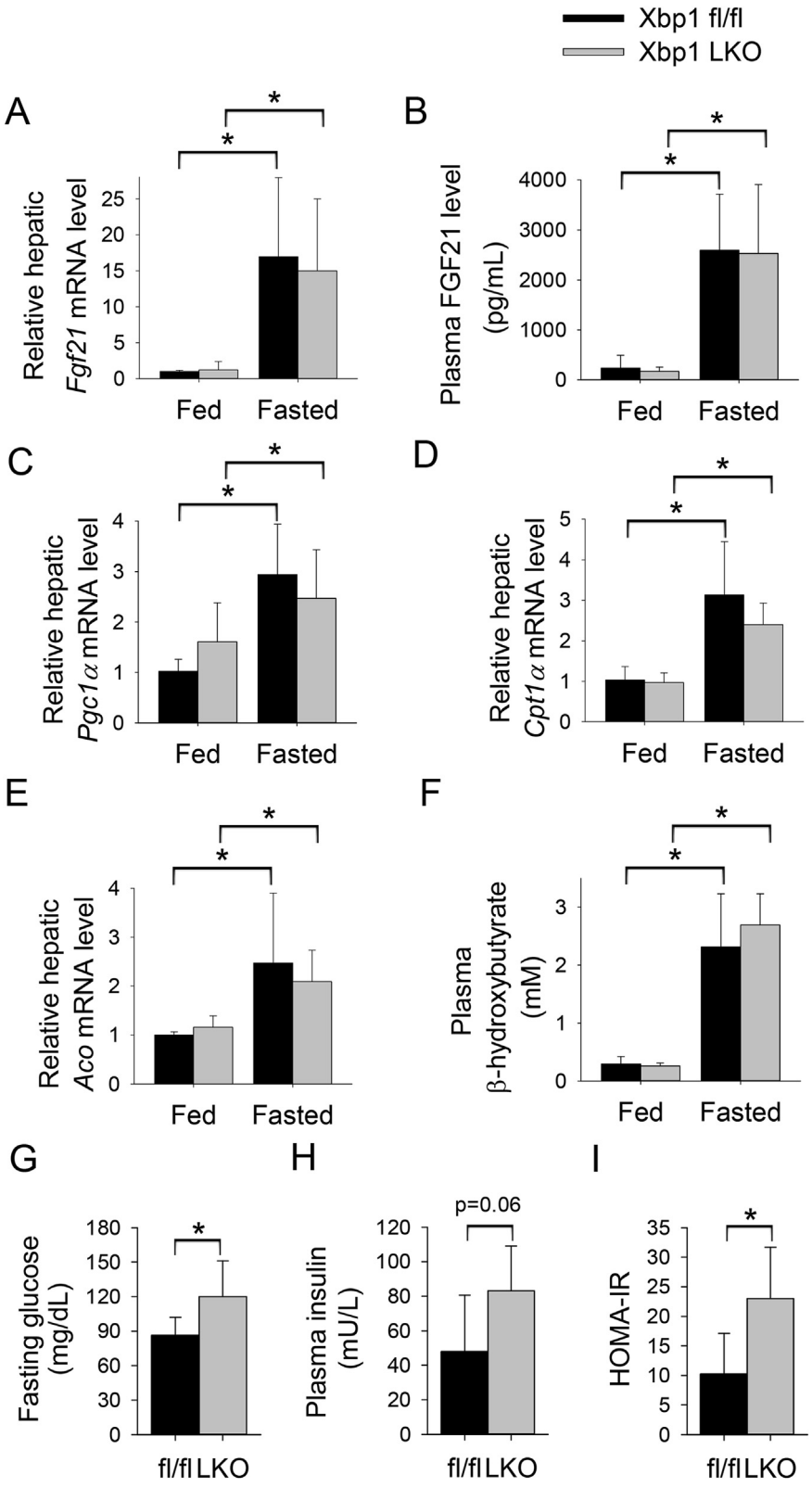

Figure 1: Hepatic *Xbp1* is not required for fasting-induced activation of FGF21. A) Hepatic *Fgf21* expression, B) plasma FGF21 concentration, C) hepatic *Pgc1α* expression, D) hepatic *Cpt1α* expression, E) hepatic *Aco* expression, F) plasma β-hydroxybutyrate concentration (mM), G) fasting whole blood glucose (mg/dL), H) fasting plasma insulin concentration (mU/L), and I) calculated homeostatic model assessment of insulin resistance (HOMA-IR) in $Xbp1^{LKO}$ or $Xbp1^{fl/fl}$ mice in the fed or fasted state. mRNA expression shown as mean ($n = 7–9$) ± SD. *$p < 0.05$.

however, whether the metabolic effects of FGF21 are mediated by Pgc1α is controversial [5,6,36]. Paralleling the induction of *Fgf21* among *Xbp1*^{LKO} mice, the hepatic expression of *Pgc1α* was appropriately induced by fasting in *Xbp1*^{LKO} mice (Figure 1C). FGF21 has also been shown to regulate other key genes controlling fatty acid β-oxidation including carnitine palmitoyl acyl-CoA transferase 1 (*Cpt1α*) and acyl-CoA-oxidase (*Aco*) [1,6]. *Xbp1*^{LKO} mice showed equivalent induction of hepatic *Cpt1α* and *Aco* expression relative to *Xbp1*$^{fl/fl}$ mice in response to fasting (Figure 1D,E). Consistent with normal induction of fatty acid oxidation genes, *Xbp1*^{LKO} and *Xbp1*$^{fl/fl}$ control mice showed an equivalent increase in plasma β-hydroxybutyrate in response to fasting (Figure 1F). These data suggest that hepatic *Xbp1* is not a major regulator of fatty acid β-oxidation in response to fasting. Complementary to its role in regulating fasting-induced fatty acid oxidation, FGF21 has also been shown to be a critical regulator of glucose metabolism [19,37–41]. Consistent with the known function of hepatic *Xbp1* in regulating glucose metabolism [42–45], we found that *Xbp1*^{LKO} mice demonstrated increased fasting blood glucose, a trend toward increased fasting plasma insulin, and an increased HOMA-IR relative to control mice (Figure 1G,H,I). Given our finding of normal FGF21 production (Figure 1A,B) in the setting of impaired glucose metabolism, we conclude that hepatic *Xbp1* regulates glucose metabolism independently of FGF21.

3.2. Hepatic *Xbp1* is not required for induction of hepatic *Fgf21* in response to ER stress

It is now well-established that ER stress induces *Fgf21* in the liver [25–27]. Having shown that hepatic *Xbp1* is not required for activation of hepatic *Fgf21* in response to fasting, we next determined whether hepatic *Xbp1* mediates ER stress-related *Fgf21* induction. *Xbp1*^{LKO} and *Xbp1*$^{fl/fl}$ mice were treated with tunicamycin for 6 h to pharmacologically induce ER stress. As expected, *Xbp1*$^{fl/fl}$ mice showed induction of spliced *Xbp1* mRNA and markedly increased hepatic expression of hepatic *Fgf21* in response to ER stress (Figure 2A,B). *Xbp1*^{LKO} mice failed to induce *Xbp1* splicing yet demonstrated normal induction of hepatic *Fgf21* in response to ER stress.

We next determined the effect of ER stress on FGF21 expression in a human cell line with a stable knockdown of XBP1 (Huh7^{shXBP1}). Huh7^{shXBP1} knockdown and Huh7^{shCON} control cells were treated with tunicamycin for 6 h. Huh7^{shCON} control cells showed induction of *XBP1* splicing and induction of *FGF21* expression in response to ER stress (Figure 2C,D). As expected, Huh7^{shXBP1} knockdown cells showed 80% suppression of *XBP1* mRNA at baseline and no induction of *XBP1* splicing in response to ER stress. Despite the failure to induce *XBP1* splicing, Huh7^{shXBP1} cells showed equal induction of *FGF21* expression compared to control cells in response to ER stress. These data indicate that *XBP1* does not mediate ER stress-induced FGF21 activation.

3.3. Deletion of hepatic *Xbp1* enhances ketogenic diet-induced hepatic *Fgf21* expression

Feeding mice a high-fat, low-carbohydrate, ketogenic diet (KD) increases circulating FGF21 levels and induces fatty acid β-oxidation pathways [1,7]. We next examined the effect of a KD on hepatic *Fgf21* expression in *Xbp1*^{LKO} and *Xbp1*$^{fl/fl}$ mice. As expected, the KD increased hepatic expression of *Fgf21* and increased plasma levels of FGF21 among *Xbp1*$^{fl/fl}$ control mice (Figure 3A,B). Interestingly, *Xbp1*^{LKO} mice fed the KD showed enhanced hepatic expression and plasma level of FGF21 relative to KD-fed *Xbp1*$^{fl/fl}$ mice. Given this unexpected finding and the potential influence of age and sex on FGF21 levels, we repeated the experiment in older, male mice. Paralleling our findings in female mice, we found that male *Xbp1*^{LKO} mice demonstrated increased hepatic expression of *Fgf21* and higher plasma levels of FGF21 compared to male *Xbp1*$^{fl/fl}$ mice when fed a KD (Figure 3A,B). Similar to our observations in the fasted state, *Xbp1*^{LKO} and *Xbp1*$^{fl/fl}$ mice showed equal induction of fatty acid oxidation genes and an equivalent increase in plasma β-hydroxybutyrate in response to a KD (Figure 3C–E).

Hepatic *Fgf21* is transcriptionally activated by several UPR signaling proteins other than *Xbp1*, most notably, ATF4 and CHOP [25,26]. We considered whether the enhanced hepatic *Fgf21* expression observed in *Xbp1*^{LKO} mice fed a KD may be due to compensatory hyperactivation of non-Xbp1-dependent elements of the UPR. Feeding a KD was

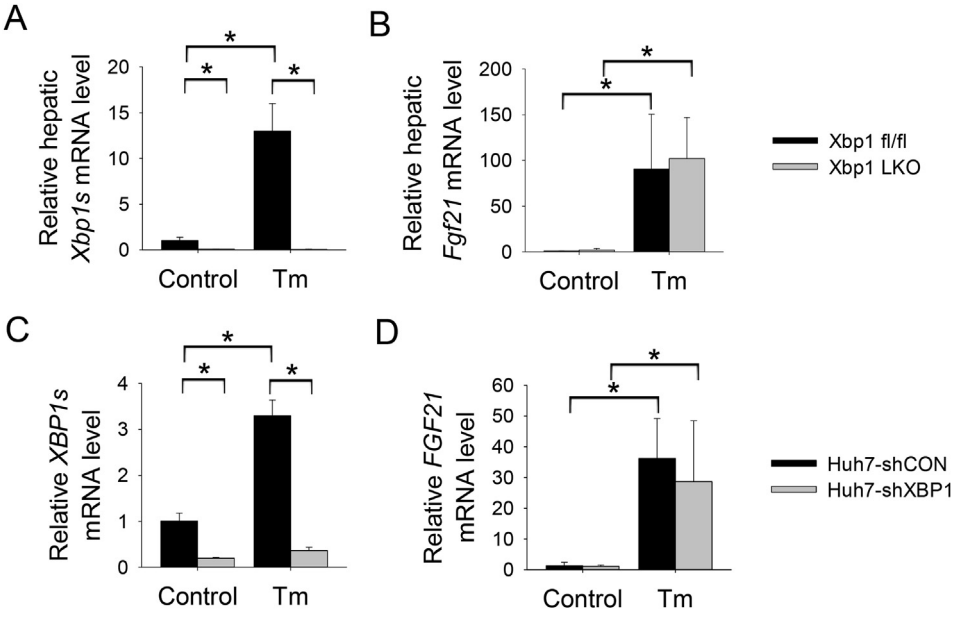

Figure 2: Hepatic *Xbp1* is not required for ER stress-induced activation of FGF21. A) Relative hepatic mRNA expression of A) *Xbp1s* and B) *Fgf21* in *Xbp1*^{LKO} or *Xbp1*$^{fl/fl}$ mice treated with tunicamycin (Tm) 0.5 mg/kg I.P. or vehicle (control) for 6 h. Mean (n = 6) ± SD. Relative mRNA expression of C) *XBP1s* and D) *FGF21* in Huh7^{shXBP1} or Huh7^{shCON} cells treated with Tm for 6 h. Mean (n = 5) ± SD. *p < 0.05.

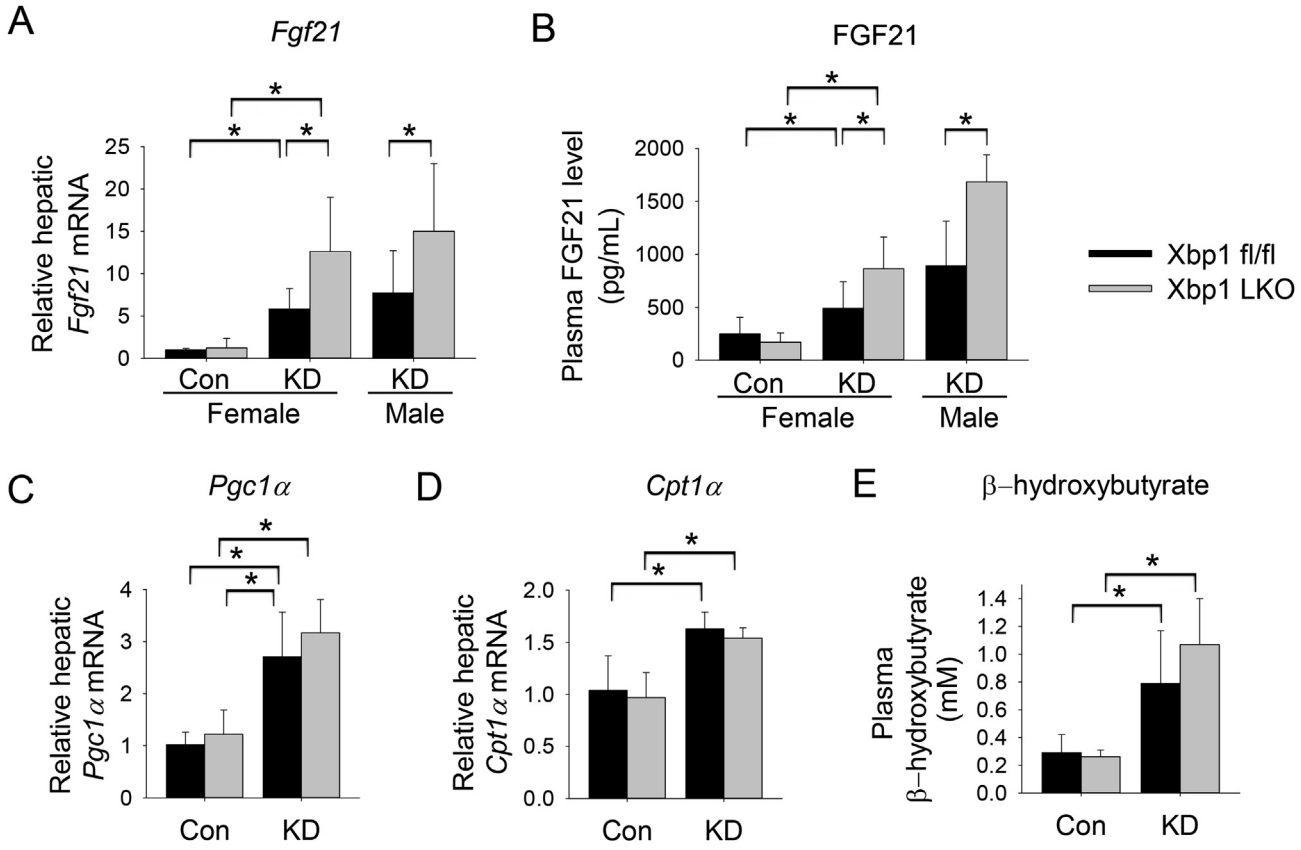

Figure 3: Deletion of hepatic *Xbp1* enhances ketogenic diet-induced activation of FGF21. A) Hepatic mRNA expression of *Fgf21*, B) plasma FGF21 concentration, C) hepatic *Pgc1α* expression, D) hepatic *Cpt1α* expression, E) plasma β-hydroxybutyrate concentration (mM) in $Xbp1^{LKO}$ or $Xbp1^{fl/fl}$ mice treated with a control or high-fat ketogenic diet (KD) for 2 weeks. Mean (n = 7–9) ± SD. *p < 0.05.

associated with activation of the UPR in the livers of $Xbp1^{fl/fl}$ control mice as evidenced by an increase in mRNA level of spliced *Xbp1*, *Atf4*, and *Chop*, as well as increased protein levels of phosphorylated IRE1α, phosphorylated eIF2α, and CHOP (Figure 4). As we and others have previously shown [29,32], $Xbp1^{LKO}$ mice showed a near-absence of hepatic spliced *Xbp1* mRNA and compensatory hyperactivation of IRE1α at baseline (Figure 4A,B). In response to a KD, $Xbp1^{LKO}$ mice showed enhanced activation of hepatic IRE1 relative to $Xbp1^{fl/fl}$ mice. $Xbp1^{LKO}$ showed a similar degree of activation of the eIF2α-ATF4 signaling cascade as evidenced by equal hepatic levels of phosphorylated eIF2α protein and *Atf4* mRNA (Figure 4A,C). On the contrary, $Xbp1^{LKO}$ mice showed markedly enhanced induction of hepatic CHOP at both the mRNA and protein level relative to $Xbp1^{fl/fl}$ mice when fed a KD (Figure 4A,D). Consistent with enhanced induction of CHOP, KD-fed $Xbp1^{LKO}$ mice showed enhanced hepatic expression of death receptor 5 (*Dr5*), a pro-apoptotic transcriptional target of CHOP (Figure 4E).

It has been shown that mice lacking hepatic *Xbp1* are protected from lipogenic diet-induced hepatic steatosis associated with suppressed expression of lipogenesis genes [29,33]. $Xbp1^{fl/fl}$ mice fed a KD for 2 weeks showed a four-fold increase in hepatic triglyceride content associated with scant hepatic steatosis on H&E staining of liver sections (Figure 4F,G). There was no overt histologic evidence of liver injury or fibrosis at this early time point. Consistent with the known effect of hepatic *Xbp1*-deletion on hepatic lipid accumulation, we found that $Xbp1^{LKO}$ mice fed a KD showed reduced hepatic steatosis (Figure 4F), modestly attenuated hepatic triglyceride accumulation (Figure 4G), and enhanced suppression of hepatic lipogenesis genes (Figure 4H) relative to $Xbp1^{fl/fl}$ mice.

4. DISCUSSION

FGF21 is a key regulator of the metabolic response to fasting and has been shown to be highly induced by ER stress [25,26]. UPR response elements have been identified within the *Fgf21* promoter, and several UPR signaling elements have been implicated in the regulation of *Fgf21* including ATF4, CHOP, and XBP1 [25–27]. While the PERK-eIF2α-ATF4 branch of the UPR has been strongly implicated in the regulation of ER stress-induced FGF21 expression, the physiologic role of XBP1 in FGF21 regulation is controversial [25,27,28,46]. We find that mice lacking hepatic *Xbp1* show normal induction of hepatic *Fgf21* in response to fasting, pharmacologic ER stress, or a ketogenic diet. Furthermore, we find that a human cell line bearing a stable knockdown of *XBP1* also demonstrates normal induction of *FGF21* in response to ER stress. Although *Xbp1* is capable of binding to UPR response elements within the *Fgf21* promoter, our data demonstrate that *Xbp1* is not required for induction of *Fgf21* under physiologic or pathophysiologic conditions.

It has been shown previously that mice bearing a liver-specific deletion of *Ire1α* show impaired activation of hepatic *Fgf21* when challenged with pharmacologic ER stress [27]. It has been speculated that the effect of *Ire1α*-deletion on hepatic *Fgf21* is a consequence of impaired *Xbp1* splicing in this model. Challenging hepatocyte-specific *Xbp1* knockout mice with stimuli that induce hepatic *Fgf21* is the most direct and definitive way to determine whether *Xbp1* is required for *Fgf21* activation *in vivo*. Our observation that mice lacking hepatic *Xbp1* demonstrate normal induction of *Fgf21* in response to fasting, ER stress, and a ketogenic diet clearly demonstrates that hepatic *Xbp1* is not necessary for

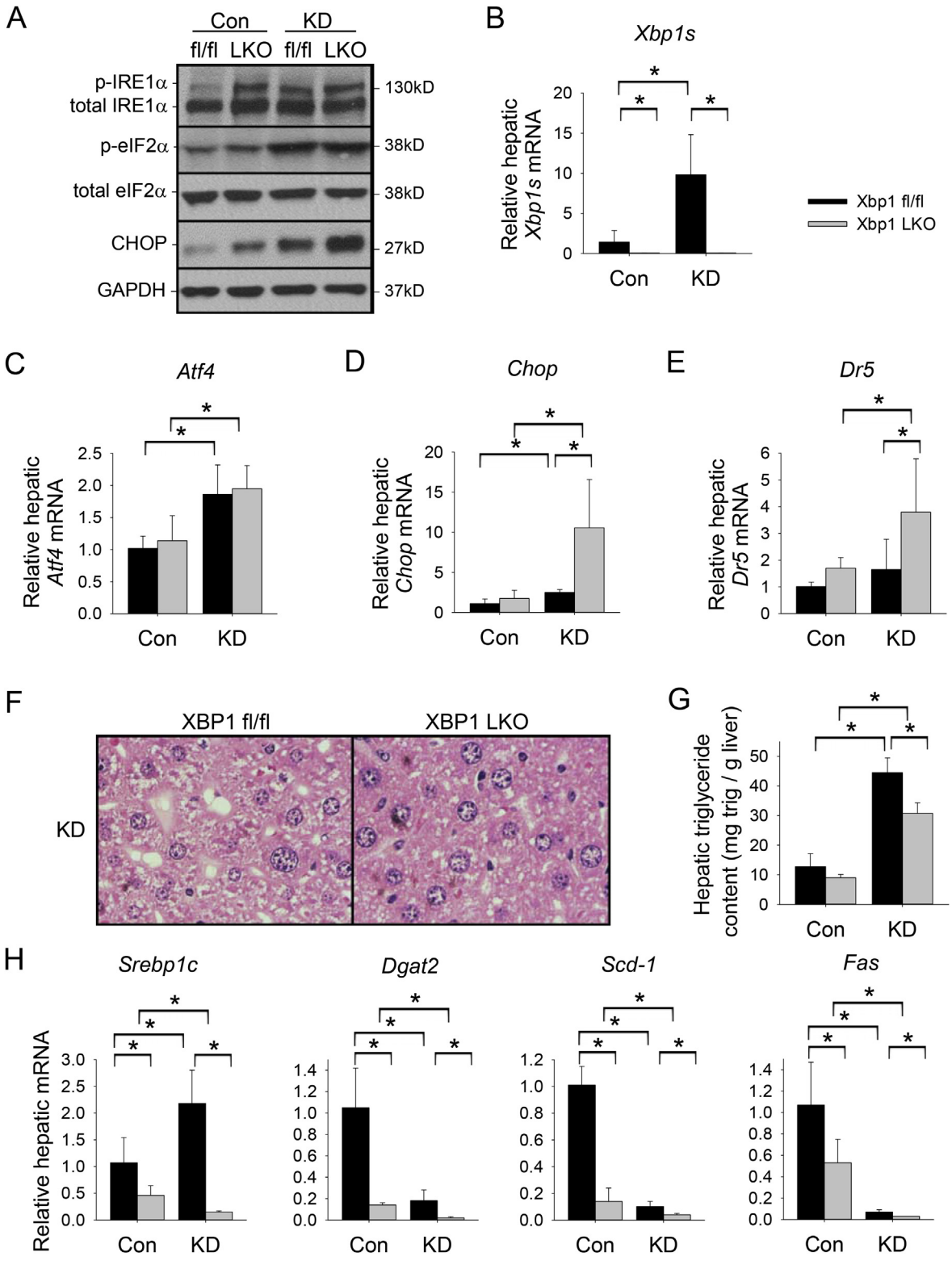

Figure 4: Deletion of hepatic *Xbp1* enhances ketogenic diet-induced UPR activation. A) Hepatic protein levels of total and phosphorylated IRE1α, total and phosphorylated eIF2α, CHOP, and GAPDH, hepatic mRNA expression of B) *Xbp1s*, C) *Atf4*, D) *Chop*, and E) *Dr5*, F) representative Oil Red O stained liver sections, G) quantification of hepatic triglyceride content (mg triglyceride per gram liver), and H) relative hepatic mRNA expression of lipogenesis genes in $Xbp1^{LKO}$ or $Xbp1^{fl/fl}$ mice treated with a control or high-fat ketogenic diet (KD) for 2 weeks. Representative western blot is of pooled samples (n = 6–7). mRNA expression shown as mean (n = 6–7) ± SD. *p < 0.05.

induction of *Fgf21 in vivo*. Furthermore, these data suggest that the effect of *Ire1α*-deletion on *Fgf21* expression is not a function of XBP1.

In addition to demonstrating that hepatic *Xbp1* does not regulate hepatic *Fgf21* expression *in vivo*, we also show that *XBP1* does not regulate ER stress-induced *FGF21* expression *in vitro*. Although consensus exists that XBP1 is capable of binding to the FGF21 promoter, conflicting data exist as to whether XBP1 is required for FGF21 activation *in vitro*. Jiang et al. showed that overexpression of *XBP1s* in primary hepatocytes induces *FGF21* expression whereas knockdown of *XBP1* blunts *FGF21* induction [27]. On the contrary, Kim et al. reported that overexpression of *Xbp1s* does not induce *Fgf21* in mouse embryonic fibroblasts (MEFs) [28]. Furthermore, they demonstrate that *Xbp1*-/- MEF cells show normal induction of FGF21 in response to pharmacologic ER stress whereas cells lacking PERK, eIF2α, or ATF4 demonstrate impaired FGF21 induction [28]. Similarly, others have concluded that the PERK-eIF2α-ATF4 branch, not the IRE1α-XBP1 branch, of the UPR is the major mediator of FGF21 induction under conditions of cellular stress *in vitro* [25]. Our data support the conclusion that XBP1 is not a major mediator of ER stress-induced FGF21 transcription *in vitro*.

Unexpectedly we find that not only is hepatic *Xbp1* not required for *Fgf21* induction but deletion of hepatic *Xbp1* actually may enhance ketogenic diet-induced FGF21 elevation. In exploring a potential mechanism for this finding, we considered whether a heightened stress response in the livers of $Xbp1^{LKO}$ mice may be the cause of enhanced hepatic *Fgf21* expression. Feeding mice a ketogenic diet induced ER stress, and deletion of hepatic *Xbp1* enhanced ketogenic diet-induced UPR activation. In particular, we found that ketogenic diet-fed $Xbp1^{LKO}$ mice showed markedly enhanced induction of hepatic CHOP, a known regulator of *Fgf21* [26]. We speculate that the enhanced hepatic stress response observed among $Xbp1^{LKO}$ mice fed a ketogenic diet underlies the enhanced *Fgf21* induction. Of note, the degree of FGF21 elevation we observed among the ketogenic diet-fed mice was less than what has been reported under other experimental conditions. This may be due in part to differing composition of the ketogenic diet; for example, ketogenic diets with lower protein content (e.g. Bioserv F3666) have been shown to induce FGF21 to a greater degree [47]. Furthermore, FGF21 is subject to circadian regulation and varying light/dark cycles among animal facilities as well as the time of blood collection could influence the degree of FGF21 elevation observed [48,49].

Consistent with the known effect of hepatic *Xbp1* in regulating *de novo* hepatic lipogenesis in response to lipogenic diets, we find that mice lacking hepatic *Xbp1* have a modestly reduced hepatic triglyceride content and suppression of lipogenesis genes in response to a high-fat, ketogenic diet [29,33]. In addition to suppression of *de novo* lipogenesis, enhanced induction of FGF21 could also contribute to the observed reduction in hepatic lipid content among ketogenic diet-fed $Xbp1^{LKO}$ mice. Moreover, induction of FGF21 has become a well-recognized feature of NAFLD in both humans and mice and is thought to be a compensatory response aimed at reducing hepatic lipid accumulation [10,11,13,16,17]. The factors that drive FGF21 expression in a steatotic liver are not fully understood, but ER stress may be a contributing factor. Furthermore, given the role of ER stress in promoting NAFLD, it is an intriguing hypothesis that induction of FGF21 by ER stress may be an adaptive response to counteract hepatic lipid accumulation. Of note, the degree of hepatic steatosis induced by the high-fat ketogenic diet was modest relative to other commonly administered lipogenic diets, likely due to the dietary composition and short-duration of feeding. Additional studies with prolonged exposure to lipogenic diets are warranted to fully characterize the interplay between ER stress, FGF21, and NAFLD.

FGF21 has emerged as a major mediator of metabolic diseases and a highly promising target for therapeutic intervention in obesity, diabetes, and fatty liver disease. Understanding the interaction between ER stress and FGF21 may have implications for the development of metabolic disease. Moreover, discerning the relevant signals that drive FGF21 expression *in vivo* may uncover novel targets for obesity, diabetes and NAFLD. Herein, we provide definitive evidence that hepatic *Xbp1* is not required for induction of hepatic *Fgf21* under physiologic and pathophysiologic conditions *in vivo*.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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