FGF21 Alleviates Hepatic Endoplasmic Reticulum Stress under Physiological Conditions

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Summary Fibroblast growth factor 21 (FGF21), a member of the endocrine FGF subfamily (also known as the FGF19 subfamily), is mainly produced by the liver. FGF21 is secreted into the blood circulation, after which it acts on target tissues through a cell-surface FGF receptor (FGFR) together with its coregulator, Klotho (1). In adipose tissues, FGF21 induces lipolysis and glucose uptake by activation of hormone sensitive lipase and glucose transporter 4, respectively (2, 3). Some hepatic energy metabolic pathways, including ketogenesis and gluconeogenesis, are also regulated by FGF21 in response to fasting. Expression of FGF21 is transcriptionally regulated during fasting via peroxisome proliferator-activated receptor α (PPARα), a nuclear hormone receptor (2, 4). More importantly, several studies revealed that FGF21 improves whole-body insulin sensitivity, inhibits high-fat diet-induced body weight gain and reduces fatty liver disease. Thus, FGF21 is an attractive target molecule for the prevention of metabolic diseases.

Endoplasmic reticulum (ER) stress is triggered by excess accumulation of either unfolded or misfolded proteins in the ER, which in turn activates three ER membrane proteins: activating transcription factor 6α (ATF6α), inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like ER kinase (PERK). During ER stress, activation of PERK results in the phosphorylation of eukaryotic initiation factor 2α (eIF2α) and translational activation of activating transcription factor 4 (ATF4). Subsequently, ATF4 induces a series of target genes involved in amino acid metabolism, redox balance and apoptosis (5). Recent studies revealed that expression of FGF21 is induced in response to ER stress and amino acid deprivation via activation of ATF4 (6–11). The promoter region of FGF21 contains three response elements for ATF4, suggesting FGF21 is a direct target of ATF4 (12).

Because FGF21 expression is induced in response to ER stress, FGF21 may work as a negative feedback molecule against ER stress. A recent study showed that administration of recombinant FGF21 protein suppressed drug-induced ER stress in the liver (9). However, no study has yet described the effects of endogenous FGF21 on ER stress. Furthermore, the function of FGF21 on physiological ER stress remains unknown. Here, we examined the effects of endogenous FGF21 on both drug-induced and physiological ER stress using FGF21 knockout (KO) mice. We also overexpressed FGF21 using a recombinant

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adenovirus for a comparison with endogenous FGF21.

MATERIALS AND METHODS

Materials. Tunicamycin was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Quantikine ELISA Mouse/Rat FGF-21 was purchased from R&D Systems (Minneapolis, MN).

Animal experiments. Male (6-wk-old) C57BL/6J mice were purchased from CLEA Japan, Inc. FGF21 KO mice were described previously (13). Mice were housed in the animal care facility at 23˚C and were allowed free access to food (Labo MR Stock; Nosan Corporation Bio Department) and water and were maintained on a 12 h light/dark cycle. For tunicamycin treatment, mice were orally or intraperitoneally administered with either vehicle (Veh; 150 mM sucrose solution) or tunicamycin (Tm; 2.5 mg/kg body weight) was administered orally. At 4 h after administration, the mice were sacrificed. (A) Serum FGF21 concentrations were determined by ELISA. (B–D) Total RNA was isolated from the liver and mRNA levels of representative target genes (B, C) or other target genes (D) of ER stress were measured using quantitative RT-PCR. All data are means±SE (n=3–5). One-way ANOVA followed by Bonferroni post hoc analysis was performed. Different superscript letters denote statistical significance (p<0.05).

β-conglycinin diet after overnight fasting, as previously described (14). The liver was rapidly excised, frozen in liquid nitrogen and stored at −80˚C until further processing. Blood samples were obtained and plasma was separated and stored at −80˚C until being processed further. All experiments were approved by and performed according to the Animal Usage Committee of The University of Tokyo (the ethical permit number: P11-567, P15-117, P17-119). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Preparation of recombinant adenovirus. Adenovirus expression plasmids for the expression of His-tagged mouse FGF21 were generated using a ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) and mammalian expression plasmids of mouse FGF21 (a gift from Dr. Takeshi Inagaki) as a template. For animal experiments, adenoviruses were amplified in 293A cells and purified using cesium chloride gradient ultracentrifugation and gel filtration on Sepharose CL-4B columns (GE Healthcare, Pittsburgh, PA) as previously reported (15).

Real-time PCR analysis. Total RNA was extracted from mouse liver using ISOGEN (Nippon Gene) accord-
ing to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized and amplified from 2 μg total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (RT-PCR) (SYBR green) analysis was performed on an Applied Biosystems StepOnePlus instrument. Relative mRNA levels were normalized to the acidic ribosomal phosphoprotein P0 (36B4) or 18S transcript. The primer sequence sets used were as follows: 36B4, 5′-CTGATCATCCACGAGTTGT-3′ and 5′-CCAGGAAGGCCCTGACCTTT-3′; FGF21, 5′-CTCTCTAGTTTCCTTGCAACAG-3′ and 5′-AAAGCTGCAAGGCTCAGGAT-3′; TRB3, 5′-GACTTGTGGCCCT-3′; CHOP, 5′-AAATAAAGGCCCCATGCTGAA-3′ and 5′-CTATTGGCTACCGAGTGTGT-3′; BiP, 5′-CTCGGAGACACAACGTTA-3′ and 5′-AGCTTCTCTTTGACACATGT-3′; XBP1s, 5′-GTGTCAGGATTTTCATGTCACCAT-3′ and 5′-CTGCCTTTTCACCTTGGAGAC-3′; Herpud1, 5′-GCTGGAC-3′; TNFα, 5′-CTTGGAAAGTGTCAGCCGGACAACTCTAAT-3′ and 5′-CATG-3′; ERdj4, 5′-CGAAGTCAAACTCTTTCAGATCCATT-3′ and 5′-TCAGGAT-3′; ATF4, 5′-AGGT-TCTTGCCAACAG-3′; FGF21, 5′-CCTCTCCAGGAAGGCCTTGACCTTT-3′ and 5′-TCCTGCTTAAATAAAAGCCCTGATGCTGAA-3′.

**Statistical analysis.** Results are expressed as mean ± SE. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis was used to compare more than two groups. Differences were considered significant at p<0.05.

**RESULTS**

**FGF21 overexpression reduces tunicamycin-induced ER stress**

FGF21 is a target gene of ATF4, which is activated by ER stress (6–8, 10, 12). As FGF21 overexpression improved several metabolic parameters, we first examined the effect of FGF21 overexpression on hepatic ER stress using an FGF21-expressing adenovirus. Mice injected with a recombinant adenovirus (mouse FGF21 [Ad-FGF21] or control LacZ [Ad-LacZ]) were orally administered with either vehicle or tunicamycin, an ER stress inducer. Marked increases in plasma and hepatic mRNA levels of FGF21 were observed after both tunicamycin treatment and injection of FGF21-expressing adenovirus (Fig. 1A and B). The expression of typical marker genes of ER stress, including Bip, XBP1s, TRB3, CHOP, and genes related to inflammation (D), were measured from the liver and mRNA levels of representative target genes (A, B) or other target genes (C) of ER stress, and genes related to inflammation (D), were measured by quantitative RT-PCR. All data are means ± SE (n=5).

One-way ANOVA followed by Bonferroni post hoc analysis was performed. Different superscript letters denote statistical significance (p<0.05).

![Fig. 2. Rescue of FGF21 in null mice potently alleviates tunicamycin-induced ER stress](image-url)

FGF21 KO virus were injected intravenously with recombinant adenovirus for control LacZ (Ad-LacZ) or mouse FGF21 (Ad-FGF21). After 6 d, vehicle (Veh; 150 μl sucrose solution) or tunicamycin (Tm; 2.5 mg/kg body weight) were administered orally. At 4 h after administration, plasma and livers were collected. (A–D) Total RNA was isolated from the liver and mRNA levels of representative target genes (A, B) or other target genes (C) of ER stress, and genes related to inflammation (D), were measured by quantitative RT-PCR. All data are means ± SE (n=5).

One-way ANOVA followed by Bonferroni post hoc analysis was performed. Different superscript letters denote statistical significance (p<0.05).

In control FGF21-deficient mice (Ad-LacZ Tm), the expression of ER stress marker genes was significantly increased upon tunicamycin administration compared with wild-type (WT) control mice (Ad-LacZ veh, Fig. 2B and C). Overexpression of FGF21 almost completely reduced tunicamycin-induced gene expression (Fig. 2B and C). Among gene sets for inflammation and apoptosis, expression of the apoptotic CHOP gene, also a target of FGF21, was significantly repressed by FGF21 (Fig. 2D).
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2D). Together, these results show FGF21 overexpression exerts a strong inhibitory effect on drug-induced ER stress.

Drug-induced ER stress is enhanced by FGF21 deficiency

To investigate the effect of endogenous FGF21 on ER stress, WT and FGF21-deficient mice were administrated tunicamycin orally. Plasma and hepatic total RNA was collected 4 h after oral administration. Marked increases in plasma and hepatic FGF21 mRNA levels (Fig. 3A and B) and ER stress marker gene expression (Fig. 3C and D) were observed in WT mice treated with tunicamycin. During ER stress, FGF21 deficiency significantly enhanced expression of several ER stress target genes such as BiP and Derl3. Although not statistically significant, a trend for FGF21 null mice toward an increase in ER stress target gene expression was observed (Fig. 3C–E). These results suggest that FGF21 deficiency augments tunicamycin-induced ER stress gene expression in the liver.

FGF21 reduces postprandial ER stress

ER stress is triggered not only by drugs and pathological conditions but also physiological conditions such as the postprandial state and a secretagogue response in pancreatic acinar cells (16, 17). Moreover, in the liver, phosphorylation of eIF2α, which in turn promotes ATF4 translation, has been reported to rise during refeeding after fasting (18). Plasma FGF21 and hepatic FGF21 mRNA levels are known to increase during fasting via PPARα (2, 4). Although FGF21 gene expression is reduced by refeeding, a significant amount of circulating FGF21 was reported during the postprandial state (19). Based on these reports, we hypothesized that FGF21 may regulate postprandial ER stress. To test this hypothesis, WT mice were injected with a recombinant...
adenovirus expressing FGF21 or a control LacZ, followed by fasting–refeeding experiments. Upon refeeding, FGF21 expression decreased, but expression of ER stress markers increased (Fig. 4A–C). FGF21 overexpression resulted in a significant reduction of BiP expression. Expression of other ER stress marker genes, including ERdj4 and HRD1, was not significantly changed. Similar experiments using FGF21 null mice (Fig. 5A) revealed that rescue of FGF21 expression clearly reduced expression of increased ER stress target genes (Fig. 5B and C). Interestingly, expression of XBP-1s was not affected by FGF21 (Figs. 4B and 5B). To investigate the effect of endogenous FGF21 in more detail, fasting–refeeding experiments were performed using WT and FGF21-deficient mice. Consistent with previous studies (2, 19), detectable levels of plasma FGF21 were observed even during refeeding, although hepatic FGF21 mRNA levels were significantly decreased (Fig. 6A and B). In FGF21-deficient mice, expression of a subset of ER stress marker genes was higher than that in WT mice (Fig. 6C and D), suggesting a significant role of endogenous FGF21 in controlling postprandial ER stress.

*Increased endogenous production of FGF21 by β-conglycinin ingestion reduces postprandial ER stress*

We recently reported that ingestion of β-conglycinin, a soy protein, resulted in a marked induction of hepatic FGF21 expression via ATF4 activation (14). To examine whether increased endogenous production of FGF21 by β-conglycinin significantly reduced postprandial ER stress, we refeed mice with either normal or β-conglycinin-containing chow after overnight fasting. Consistent with a previous study (14), plasma FGF21 and hepatic FGF21 mRNA levels were greatly increased by β-conglycinin ingestion (Fig. 7A and B). Expression of other ATF4 target genes, including TRB3 and CHOP, was also significantly increased (Fig. 7C and D). As observed in experiments using an FGF21-expressing adenovirus (Figs. 4B and 5B), the expression of BiP, an ER stress-regulated gene independent of ATF4, was significantly decreased by β-conglycinin ingestion (Fig. 7C). These results suggest that increasing endogenous FGF21 with β-conglycinin potently reduces postprandial ER stress.

**DISCUSSION**

In this study, we found that FGF21 alleviated both drug-induced pathological ER stress and physiolog-
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Cal ER stress triggered by refeeding after fasting. Notably, endogenous FGF21 was effective in reducing the ER stress response under these conditions. In addition, increased circulating FGF21 induced by intake of the soy protein β-conglycinin, as opposed to casein, resulted in a significant reduction of the postprandial ER stress response.

Although the effects of FGF21 overexpression on ER stress were obviously potent, we found that endogenous FGF21 was also potentially capable of alleviating ER stress. When FGF21 expression was strongly induced in response to chemical ER stress inducers, such as tunicamycin, a rapid decline in increased FGF21 expression was observed even in the presence of these chemical inducers (data not shown). In addition, it is well known that circulating FGF21 protein is labile and has a short half-life (20, 21). In contrast, adenovirus-mediated expression is thought to keep its circulating concentration steady at relatively high levels. This appears to account for the observed potent effects of FGF21 overexpression and a slight positive effect of endogenous FGF21 in WT mice compared with FGF21-deficient mice. Importantly, this study demonstrates that the increase in circulating FGF21 seen after β-conglycinin ingestion, which increases hepatic FGF21 gene expression via the transcription factor ATF4, was potent enough to alleviate the postprandial ER stress response in the liver.

ER stress activates three downstream effectors: PERK–ATF4, IRE1–XBP1 and ATF6α. It has been reported that FGF21 gene expression is directly regulated by ATF4 (7, 8, 12, 22) and potentially XBP-1 (9). A previous study reported that FGF21 repressed ER stress by inhibiting phosphorylation of eIF2α, an upstream activating factor of ATF4 (9). This implies that the ER stress–ATF4–FGF21 axis creates a negative feedback loop. However, our results showed that FGF21 inhibits induction of target genes of all three downstream effectors. Therefore, FGF21 appears to be a regulatory factor in the proteostasis network and not merely an end-point regulator in the negative feedback loop. ER stress is thought to be caused not only by accumulation of excess unfolded or misfolded proteins in the ER but also pathogenetic conditions, such as obesity and diabetes (23–27), and physiological conditions such as fasting and refeeding (18). In addition, treatment with chemical chaperones that reduce ER stress improved several metabolic parameters.
including insulin sensitivity (28). Based on our data, together with these previous findings, we propose that FGF21 plays a critical role in the proteostasis network to regulate protein homeostasis. During the postprandial state, FGF21 overexpression did not alter XBP-1s generation, whereas expression of other ER stress target genes was repressed (Figs. 4 and 5). This seems likely due to the induction of glucose-dependent XBP-1s in addition to ER stress (29).

Previous KO gene studies showed that the absence of ER stress-sensing proteins, including ATF6α, caused hepatic steatosis (30, 31). It was also reported that ER stress-induced hepatic steatosis was alleviated by the administration of recombinant FGF21 protein (9). Our data in FGF21 KO mice support these studies and provide more powerful evidence of the relationship between FGF21 and ER stress.

It was initially assumed that FGF21 secreted from the liver acts on adipose tissues in an endocrine manner (2, 3). However, previous publications have concluded that FGF21 regulates fatty acid metabolism through the FGFR/βKlotho signaling pathway in the liver (2, 4, 9), suggesting circulating FGF21 may function in an endocrine and autocrine/paracrine manner. These studies seem to support the idea that the inhibitory effects of endogenous FGF21 on ER stress in the liver are due to an interaction between FGF21 and its receptor, the FGFR/βKlotho complex, though this study does not show any evidence suggesting their direct interaction. Given that postprandial ER stress occurred within 3 h of refeeding after overnight fasting and was alleviated by increased circulating FGF21 in response to soy protein ingestion, it seems more likely that FGF21 directly exerts its actions in the liver, rather than indirectly via FGF21 acting on another tissue, such as adipose tissue, or unidentified circulating factors.

In summary, we demonstrate that FGF21 alleviates ER stress under pharmacological as well as physiological conditions. Because chronic ER stress has been implicated as a pathogenic determinant in some diseases, including diabetes and inflammation, it is suggested that FGF21 may function as a critical regulator to maintain protein homeostasis by reducing ER stress.

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Fig. 7. Postprandial ER stress is suppressed by β-conglycinin ingestion. WT mice were subjected to overnight fasting followed by refeeding with either chow or a β-conglycinin diet (βicon) for 3 h. (A) Plasma FGF21 levels were determined by ELISA. (B–D) Total RNA was isolated from the liver and mRNA levels of representative target genes (B, C) or other target genes (D) of ER stress were measured by quantitative RT-PCR. All data are means±SE (n=5). *p<0.05, **p<0.01.

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