Selective Regulation of FGF19 and FGF21 Expression by Cellular and Nutritional Stress

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Summary Fibroblast growth factor 19 (FGF19) and FGF21 are members of a subfamily of the FGFs called endocrine FGFs. FGF19 regulates the bile acid synthetic pathway. FGF19 expression is induced by farnesoid X receptor (FXR), a nuclear hormone receptor activated by bile acids in the small intestine. FGF21 plays an important role in lipolysis that occurs in white adipose tissue. FGF21 expression is stimulated by the nuclear fatty acid receptor peroxisome proliferator-activated receptor α (PPARα) in the liver. FGF19 and FGF21 were recently identified as targets of activating transcription factor 4 (ATF4), which is activated in response to endoplasmic reticulum (ER) stress. ATF4 is also activated by oxidative stress and amino acid deprivation. In this study, we investigated FGF19 and FGF21 expression in response to oxidative stress and amino acid deprivation. We found that FGF19 mRNA is induced by oxidative stress inducers in Caco-2 cells, which are derived from the human intestinal epithelium, and rat intestinal epithelial IEC6 cells. In contrast, ileal FGF15 expression, the rodent ortholog of human FGF19, is not increased by oxidative stress. No notable changes in expression of FGF15/19 took place under amino acid deprivation either in vitro or in vivo. In contrast, FGF21 expression is induced by oxidative stress and amino acid deprivation both in vitro and in vivo. These results indicate distinctive patterns of regulation of FGF19 expression by ER stress, and FGF21 expression by ER stress, oxidative stress, and amino acid deprivation through ATF4 activation.

Key Words FGF19, FGF21, ATF4, oxidative stress, amino acid deprivation

Fibroblast growth factor 19 (FGF19) and FGF21 belong to the endocrine FGF subfamily, which also includes FGF23. FGF19 expression is dominant in the ileum, where it is regulated by the nuclear bile acid receptor farnesoid X receptor (FXR) (1, 2). After secretion into the bloodstream, FGF19 negatively regulates hepatic bile acid metabolism through a repression of cytochrome P450 7A1 (CYP7A1), a key enzyme of bile acid synthesis. Other important functions of FGF19, like gall bladder filling, inhibition of gluconeogenesis, and stimulation of protein synthesis, have also been reported (3–5). On the other hand, hepatic FGF21 expression is regulated by peroxisome proliferator-activated receptor α (PPARα), which is activated by fatty acids (6–8). FGF21 acts as a hepatokine; it stimulates glucose uptake and lipolysis in white adipose tissue, as well as ketone body production and hepatic gluconeogenesis in the liver (7, 9, 10). It is interesting that both FGF19 and FGF21 show beneficial effects on obesity and insulin sensitivity (11–14). Recent studies revealed that a synthetic FGF21 analog improves lipid metabolism in obese humans and monkeys with type 2 diabetes (15, 16). These reports suggest FGF19 and FGF21 are attractive therapeutic targets for obesity and diabetes.

Our group (17) and other groups (18–21) have reported that FGF19 and FGF21 are novel responsive genes of endoplasmic reticulum (ER) stress, which is caused by excess accumulation of unfolded proteins in the ER. Upon ER stress, the unfolded protein response (UPR) pathway, comprising activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1), and protein kinase RNA-like ER kinase (PERK), is activated, resulting in the induction of chaperones and proteins for ER-associated degradation (22). FGF19 and FGF21 have been identified as targets of ATF4, a transcription factor downstream of PERK. Functional ATF4-binding sites are found in the promoter region of these genes. In addition to ER stress, ATF4 is also activated by oxidative stress, amino acid deprivation, and viral infection (23).

While the induction of FGF21 expression by oxidative stress and amino acid deprivations have been reported (18–20), it is unknown whether FGF19 expression is regulated by these stimuli. In this study, we investigated the elevation of FGF19 and FGF21 expression by ATF4 stimulators other than those that cause ER stress. FGF19 expression is induced by oxidative stress in vitro, but not in vivo. Amino acid deprivation does not regulate expression of FGF19 either in vitro or in vivo. In contrast, FGF21 responds to oxidative stress and amino acid deprivation both in vitro and in vivo. Our findings reveal a selective regulation of FGF19 and FGF21 expression...
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MATERIALS AND METHODS

Materials. Sodium arsenite was obtained from Nakalai Tesque, Kyoto, Japan. Hydrogen peroxide and amino acids were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Cell culture. Caco-2 cells were maintained in medium A (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37°C in an atmosphere containing 5% CO2. Cells that had been cultured for 14 d after confluency were considered differentiated. IEC6 cells were maintained in medium B (Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 4 μg/mL human insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37°C in an atmosphere containing 5% CO2. HepG2 cells were maintained in medium C (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37°C in an atmosphere containing 5% CO2. For amino acid experiments, cells were cultured with control medium or leucine-deficient medium, supplemented with 5% dialyzed fetal bovine serum. For control medium, 20 amino acids were added to the amino acid-free medium (24, 25), which was kindly provided by Ajinomoto, Kawasaki, Japan. The amino acid concentrations were as follows: 0.08 mM Trp; 0.2 mM Cys, Met, His; 0.4 mM Gly, Ala, Ser, Asn, Glu, Asp, Phe, Tyr, Arg, Pro; 0.8 mM Thr, Val, Leu, Ile, Lys; 4 mM Gln.

Animals. Male C57BL/6 mice were purchased from Clea Japan Inc., Tokyo, Japan. Mice were maintained on a 12 h light/dark cycle in the animal care facility of The University of Tokyo. Mice were fed standard chow (Labo MR Stock; Nosen Corp. Bio Department, Yokohama, Japan) ad libitum and had free access to water. For arsenite treatments, either vehicle or arsenite was orally administered (400 μg/mouse). Two hours after administration, mice were humanely sacrificed, and the liver and ileal mucosa was harvested, and then total RNA was isolated. mRNA levels, which were determined by quantitative real-time PCR, are presented as relative expression after normalization to 36B4 mRNA (A and C) or RPL19 mRNA (B). Relative mRNA levels in vehicle-treated mice were set to 1. All data are presented as mean±SD (n= 3). * p<0.05, ** p<0.01.

Fig. 1. Oxidative stress induces FGF15/19 expression in vitro, but not in vivo. (A) Caco-2 cells were differentiated by continuous culture for 14 d after they had reached confluency. Cells were then treated with vehicle (Veh) or 30 μM arsenite (A) for 8 h, after which total RNA was isolated. (B) IEC6 cells were treated with vehicle (Veh) or 30 μM arsenite for 4 h, after which total RNA was isolated. (C) Wild-type male mice received oral administration of vehicle (Veh) or arsenite (400 μg/mouse). Two hours after administration, ileal mucosa was harvested, and then total RNA was isolated. mRNA levels, which were determined by quantitative real-time PCR, are presented as relative expression after normalization to 36B4 mRNA (A and C) or RPL19 mRNA (B). Relative mRNA levels in vehicle-treated mice were set to 1. All data are presented as mean±SD (n= 3). * p<0.05, ** p<0.01.
Real-time PCR. Total RNA was extracted using IsoGen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesize and amplify cDNA from total RNA. Quantitative real-time PCR was performed with an Applied Biosystems 7000 sequence detection system. Relative mRNA levels were determined by normalizing to the acidic ribosomal phosphoprotein P0 (36B4) transcript (mouse and human) or the ribosomal protein L19 (RPL19) transcript (rat). The sequences of the primer sets used were previously shown (7, 17, 26–28). Sequences of other primer sets used in this study were as follows: mouse asparagine synthetase (ASNS), 5′-CAAGGAGGCCCAAGTTCAGTAT-3′ and 5′-GGCTGTCTCCATCTGTTGTG-3′; rat FGF21, 5′-CACCCGAGTAGAAAGTCT-3′ and 5′-ATGAGAGTTCCATCTGGTTG-3′; rat RPL19, 5′-CTCATGGAGCACATCCACAA-3′ and 5′-TGGTACGACCAGTCTTTG-3′; rat C/EBP homologous protein (CHOP), 5′-TCCTGTCCTCAGATG-3′ and 5′-ACGGGTGTTGTCCTCT-3′; and rat FGF15, 5′-ACGGGCTGATTCGCTACTC-3′. PCR primer sequences for rat RPL19 and CHOP were provided by Dr. David J. Mangelsdorf and Dr. Steven A. Kliewer.

Statistical analysis. Results are presented as mean±SD and were compared between groups using Student’s t-tests.

RESULTS

FGF19 expression is induced by oxidative stress in vitro, but not in vivo

A human intestinal epithelial cell line (Caco-2) was used to investigate the effects of oxidative stress and amino acid deprivation on FGF19 expression. When differentiated Caco-2 cells were cultured with arsenite, an inducer of oxidative stress, FGF19 and other ATF4 targets, activating transcription factor 3 (ATF3) and C/EBP homologous protein (CHOP), were strongly upregulated (Fig. 1A). Arsenite treatment also increased ATF4 mRNA, indicating ATF4 activation (29). Another oxidative stress inducer, hydrogen peroxide, induced FGF19 expression (data not shown). In order to examine the response to oxidative stress in rodent cells, IEC6 cells were cultured with arsenite. Increased expression of ATF4 and its target genes including FGF15, a rodent ortholog of FGF19, was observed (Fig. 1B). Next, we examined whether oxidative stress regulates FGF15 expression in vivo. Mice were treated with arsenite by oral gavage. After 2 h of administration, total RNA was extracted from the ileal mucosa, in which FGF15 is highly expressed. FGF15 expression was not affected by arsenite. Additional experiments demonstrated that ileal FGF15 expression is not altered 1, 2, or 4 h after oral gavage with arsenite (data not shown). However, a significant increase in the expression of ATF4 and its target genes, ATF3 and CHOP, was observed (Fig. 1C). Moreover, expression of the gene encoding heme oxy-
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genase-1, a target of NF-E2-related factor 2 (NRF2) that is also activated by oxidative stress, was induced by arsenite (data not shown). These results indicate that FGF19 expression is regulated by oxidative stress in vitro, but not in vivo.

Regulation of FGF21 expression by oxidative stress was examined using HepG2 cells. FGF21 expression was strongly induced (Fig. 2A). The hepatic FGF21 mRNA level was also increased by oral gavage with arsenite (Fig. 2B). These data demonstrate that FGF21 expression is regulated by oxidative stress both in vitro and in vivo.

**FGF19 expression is not induced by amino acid deprivation.**

In order to determine whether amino acid deprivation increases FGF19 expression, differentiated Caco-2 cells were cultured for 8 h in a leucine-deficient medium, which has been reported to activate ATF4 in mouse embryonic stem cells (30). Leucine deprivation had no effect on the expression of FGF19 or ATF4 target genes including those encoding asparagine synthetase (ASNS) and CHOP (Fig. 3A). We next examined deprivation of all essential amino acids (valine, leucine, isoleucine, methionine, tryptophan, lysine, phenylalanine, threonine, and histidine). FGF19 expression was not increased by essential amino acid-deficient medium (data not shown). We also did not observe an increase in FGF19 expression when cells were treated with leucine- or essential amino acid-deficient media for 4 or 20 h (data not shown). Furthermore, when differentiated Caco-2 cells were treated with amino acid-free Krebs–Ringer bicarbonate buffer (31), no increase in FGF19 expression was observed (data not shown). Leucine deprivation also had no effect on the expression of FGF15 in IEC6 cells, whereas the expression of other ATF4 target genes, ASNS and CHOP, was increased (Fig. 3B). To investigate the effect of amino acid deprivation in vivo, mice were fed a leucine-deficient diet for 1 wk. Ileal levels of FGF15 mRNA and the mRNA of other ATF4 target genes were not significantly influenced by leucine deprivation (Fig. 3C). We also observed that the expression of ATF4 target genes was not altered in the jejunum or duodenum of mice treated with the leucine-deficient diet (data not shown). These results clearly show that amino acid deprivation does not stimulate expression of FGF15/19 either in vitro or in vivo. Regulation of FGF21 expression by amino acid deprivation was analyzed using HepG2 cells.

![Fig. 3](image-url)
Fgf21 expression as well as that of other ATF4 target genes was markedly increased by leucine-deficient media (Fig. 4A). Similarly, when mice were fed a leucine-deficient diet for 1 wk, the hepatic Fgf21 mRNA level was significantly increased (Fig. 4B). These data show that Fgf21 expression increases in response to amino acid deprivation both in vitro and in vivo.

DISCUSSION

In the present study, we investigated the effects of oxidative stress and amino acid deprivation, which activate ATF4 as well as ER stress, on Fgf19 and Fgf21 expression. We demonstrated that oxidative stress enhances Fgf21 expression in vitro and in vivo. These results are consistent with previous reports (18–21). In contrast, Fgf15/19 expression is induced only in vitro. These results indicate that ileal Fgf15 expression is not responsive to oxidative stress. Upon initiation of oxidative stress, heme-regulated eIF2α kinase (HRI) promotes phosphorylation of eukaryotic initiation factor 2α (eIF2α), resulting in a selective increase in ATF4 translation (23). We observed significant HRI mRNA levels in Caco-2 cells and the ileum as well as liver (data not shown). Moreover, oxidative stress enhanced expression of ATF4 target genes other than Fgf15 in the ileum (Fig. 1C), suggesting that the HRI-eIF2α-ATF4 pathway is functional. Because among ATF4 target genes, only the transcription of Fgf15/19 is regulated by the nuclear bile acid receptor FXR (1, 2, 17), regulation of Fgf19 expression by oxidative stress may require FXR activation in addition to ATF4.

Our data show that amino acid deprivation has no effect on the expression of Fgf19 either in vitro or in vivo, whereas Fgf21 mRNA levels are increased in hepatocytes and the liver. Expression of ATF4 and its target genes except for Fgf15/19 was not affected by amino acid deprivation in Caco-2 cells or the ileum. Since we also observed that expression of ATF4 target genes was not altered in the jejunum or duodenum of mice fed a leucine-deficient diet, our results suggest that the small intestine does not respond to amino acid deprivation. This is interesting because the intestine is an essential tissue for nutrient absorption. On the other hand, mammalian target of rapamycin (mTOR), an amino acid sensor, has been reported to respond to amino acids in the intestinal villi (32). Thus, it appears that the small intestine is a tissue that responds to ingested amino acids, but not to amino acid deprivation. Fgf19 expression is induced by bile acids upon feeding (1, 2), and-secreted Fgf19 enhances hepatic protein synthesis (3). These reports suggest that the intestinal ATF4-Fgf19 pathway may be inactivated to prevent protein synthesis when amino acid levels are low, such as while fasting. Expression of ATF4 and its target genes was increased by amino acid deprivation in rat IEC6 cells, indicating a cell-selective regulation of ATF4 pathway in vitro.

In addition to the liver, Fgf21 is also expressed in adipose tissue and skeletal muscle. It is interesting that Fgf21 expression is not altered in these tissues in mice fed a leucine-deficient diet (18, 33), whereas hepatic Fgf21 is induced. This study revealed that amino acid deprivation has no effect on the expression of ATF4 target genes in the small intestine. In contrast to oxidative stress, amino acid deprivation activates ATF4 through general control non-derepressible-2 (GCN2), another eIF2α kinase (23). GCN2 has been reported to respond to amino acid deprivation in the brain (34), liver (35), macrophages (36), and mouse embryonic stem cells (30). Thus, activation of ATF4 in response to amino acid deprivation may be tissue selective. What is the mechanism of inactivation of the intestinal amino acid response through the GCN2-ATF4 pathway? A significant GCN2 mRNA is observed in the mouse ileum and Caco-2 as well as the mouse liver (data not shown). It has also been reported that GCN2 is ubiquitous in tissues (37, 38). Moreover, ER stress stimulates expression of Fgf19 and ATF4 target genes in Caco-2 cells and the mouse ileum (17), suggesting that the eIF2α-ATF4 pathway is functional. Therefore, it is unclear why the small intestine does not respond to amino acid deprivation. Further studies, including an analysis of GCN2 protein levels, are needed.

Our present study and previous reports (17–21) revealed that ileal Fgf15 mRNA is increased by ER stress, and that hepatic Fgf21 expression is induced by

Fig. 4. Amino acid deprivation induces Fgf21 expression. (A) HepG2 cells were treated with control (Con) and leucine-deficient media (−Leu) for 5 h, after which total RNA was isolated. Results are presented as mean±SD (n = 3). (B) Wild-type male mice were fed control (Con) or leucine-deficient diets (−Leu). One week after the beginning of the experiment, tissues were harvested from these mice, and then total RNA was isolated. mRNA levels, determined using quantitative real-time PCR, are presented as relative expression after normalization to 36B4 mRNA. Relative mRNA levels in vehicle-treated mice were set to 1. Results are presented as mean±SD (n = 5–6).
Selective Regulation of FGF19 and FGF21 by ATF4 by ATFSI stimulators. Stimulators of ATF4 activation selectively regulate expression of ileal FGF15/19 and hepatic FGF21.

ER stress, oxidative stress, and amino acid deprivation, among ATFSI stimulators (Fig. 5). Oxidative stress stimulates FGF19 expression only in vitro. Secreted FGF21 has been reported to ameliorate oxidative damage (39) and inhibit the expression of genes related to lipogenesis that are increased by a leucine-deficient diet (33). These observations are physiologically relevant; hence, FGF21 expression is regulated by oxidative stress and amino acid deprivation. Because FGF19 expression is not regulated by oxidative stress or amino acid deprivation in vivo, we speculate that FGF19 may not act on these pathways. Since the ER stress-ATF4 pathway stimulates FGF19 expression (17), further study should focus attention on a functional analysis of FGF19 during ER stress.

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