Fibroblast Growth Factor 21 Induces Glucose Transporter-1 Expression through Activation of the Serum Response Factor/Ets-Like Protein-1 in Adipocytes

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Background: FGF21 increases glucose uptake in adipocytes by enhancing the expression of glucose transporter-1 (GLUT1).

Results: FGF21 induces the phosphorylation of the transcription factors serum response factor (SRF) and Ets-like protein-1 (Elk-1), which in turn bind to a highly conserved cis-element within the GLUT1 gene promoter for transcriptional activation. Such a stimulatory effect of FGF21 is impaired in adipose tissue of diet-induced obese mice.

Conclusion: SRF and Elk-1 act synergistically to mediate FGF21-induced GLUT1 gene expression in adipocytes.

Significance: The findings provide new molecular insights into the metabolic actions of FGF21 in its major target tissue.

Fibroblast growth factor 21 (FGF21) is a liver-secreted endocrine factor with multiple beneficial effects on obesity-related disorders. It enhances glucose uptake by inducing the expression of glucose transporter-1 (GLUT1) in adipocytes. Here we investigated the signaling pathways that mediate FGF21-induced GLUT1 expression and glucose uptake in vitro and in animals. Quantitative real-time PCR and a luciferase reporter assay showed that FGF21 induced GLUT1 expression through transactivation. The truncation of the GLUT1 promoter from −3145 to −3105 bp, which contains two highly conserved serum response element (SRE) and E-Twenty Six (ETS) binding motif, dramatically decreased FGF21-induced promoter activity of the GLUT1 gene. A chromatin immunoprecipitation assay demonstrated that the transcription factors serum response factor (SRF) and Ets-like protein-1 (Elk-1) were recruited to the GLUT1 promoter upon FGF21 stimulation. The siRNA-mediated knockdown of either SRF or Elk-1 resulted in a marked attenuation in FGF21-induced GLUT1 expression and glucose uptake in adipocytes. In C57 lean mice, a single intravenous injection of FGF21 induced phosphorylation of Elk-1 at Ser 383 and SRF at Ser 103 and also led to the recruitment of Elk-1 and SRF to the GLUT1 promoter in epididymal fats. By contrast, such effects of in vivo FGF21 administration were blunted in high fat diet-induced obese mice. In conclusion, FGF21 induces GLUT1 expression and glucose uptake through sequential activation of ERK1/2 and SRE/Elk-1, which in turn triggers the transcriptional activation of GLUT1 in adipocytes. The impairment in this signaling pathway may contribute to FGF21 resistance in obese mice.

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2 The abbreviations used are: FGF21, fibroblast growth factor 21; GLUT1, glucose transporter-1; SRE, serum response element; ETS, E-Twenty Six; SRF, serum response factor; Elk-1, Ets-like protein-1; AMPK, AMP-activated protein kinase; SirT1, silent mating type information regulation 2 homolog 1.
Glucose Uptake in Adipocytes—Adipocytes were seeded at 2.5 × 10^5/well in 12-well plate 1 day before the assay. The cells were starved for 24 h in DMEM plus 0.5% FBS followed by stimulation with FGF21 for another 24 h. After that, the cells were switched to glucose-free DMEM medium plus FGF21 for 4 h. Then the adipocytes were washed twice with KRP buffer (15 mM HEPES, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO_4, 1.3 mM CaCl_2, 1.2 mM KH_2PO_4, 0.1% BSA), and 500 μl of fresh KRP buffer containing 2-deoxy-D-[^3H]glucose (0.1 μCi, 100 μM) was added to each well. Cytochalasin B (20 μM) was used to determine the nonspecific absorption. The glucose uptake reaction was carried out for 1 h at 37 °C, and the cells were lysed with 0.1 M NaOH and neutralized by an equal amount of HCl. The radioactivity was analyzed by liquid scintillation counting.

Construction of Luciferase Reporter Vectors Driven by the Mouse GLUT1 Promoter—The mouse GLUT1 promoter region spanning −3710 to −49 bp was amplified by PCR using mouse genomic DNA as a template and then subcloned into pGL3-Basic vector to obtain the luciferase reporter vector driven by the 3.7-kb GLUT1 promoter. The putative serum response element (SRE) and E-Twenty Six (ETS) binding site within the promoter region were mutated by mutagenesis PCR using their wild type vectors as the templates. The constructs were confirmed by DNA sequencing. The sequences of all the primers used for the vector construction are listed in supplemental Table 1.

Transient Transfection and Luciferase Assay—3T3-L1 preadipocytes were seeded at 1 × 10^5/well in 12-well plates 24 h before transfection with the luciferase reporters using JetPEI (Poluplus) according to the manufacturer’s instructions. The cells were then subjected to differentiation induction as described above followed by treatment with various concentrations of FGF21 for 24 h. After that, cells were lysed in a reporter lysis buffer, and the luciferase activity was measured using Bright-GloTM Luciferase Assay System (Promega) as described (24). The siRNA was introduced into cells by electroporation using Gene pulser Xcell (Bio-Rad). The siRNA sequences used in this study are listed in supplemental Table 2.

RNA Extraction and Real-time PCR—Total RNA from 3T3-L1 adipocytes was purified with a TRizol reagent (Invitrogen). For reverse transcription, 1 μg of the total RNA was converted to first-strand complementary DNA in 20 μl reactions using a cDNA synthesis kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green PCR MasterMix (Promega) on an Applied Biosystems Prism 7000 sequence detection system. Analysis was performed with ABI Prism 7000 SDS Software and normalized against 18S RNA. Primer sequences used for real-time PCR are listed in supplemental Table 3.

ChIP—The ChIP assay was performed as described (25) with minor modifications. 80–90% confluent 3T3-L1 adipocytes were treated with or without FGF21 for various periods followed by fixation with 1% formaldehyde for 15 min at room temperature. Cells were lysed, and chromatin was sheared by sonication at 4 °C. 25 μg of the lysates were incubated overnight at 4 °C with 2 μg of anti-SRF antibody or anti-Elk-1 antibody or rabbit non-immune IgG as the negative control followed by precipitation with ChIP grade protein G beads. The precipitates were washed extensively and eluted with the elution buffer (1%
SDS, 0.1 M NaHCO₃) at room temperature for 15 min. Input chromatin and immunoprecipitated chromatin were incubated at 65 °C overnight to reverse the cross-links. After digestion with protease K, DNA was extracted with phenol-chloroform and precipitated with ethanol. Purified DNA was analyzed by quantitative real-time PCR using primers specific to GLUT1 promoter listed in supplemental Table 3. All results were normalized to the respective input values.

Western Blot Analysis—30 μg of 3T3-L1 lysates were separated by 10% SDS-PAGE and probed with various primary antibodies as specified in each figure legend. The proteins were visualized by chemiluminescence detection. The relative intensity of the protein bands was quantified using the Multi Analyst software package (Bio-Rad) as described (26).

Animal Studies and in Vivo Protocols—6-Week-old C57BL/6 male mice obtained from the laboratory animal unit, the University of Hong Kong, were fed with either standard chow or high fat diet (Research Diet, 20 kcal % protein, 45 kcal % fat, and 35 kcal % carbohydrates) for 12 weeks to induce obesity. One day before the experiment mice were fasted for 16 h (from 5:30 p.m. to 9:30 a.m.) followed by a single intravenous injection of recombinant FGF21 (1.5 μg/100 g of body weight) or PBS as a control. At various time points after the injection, epididymal adipose tissues were collected from mice immediately after anesthesia, weighed, homogenized, and then subjected to either Western blot analysis or fixed with 1% formaldehyde. The chromatin in epididymal adipose tissue was sheared and subjected to ChIP analysis to detect the binding of Elk-1 and SRF to the endogenous GLUT1 gene as described above. All animal experimental protocols were approved by the Animal Ethics Committee of the University of Hong Kong.

Statistical Analysis—Data are expressed as the mean ± S.D. Statistical significance was determined by one-way analysis of variance or Student’s t test. In all statistical comparisons p value <0.05 was used to indicate a significant difference.

RESULTS

FGF21 Induces Glucose Uptake via the ERK1/2 Signaling Pathway in Adipocytes—Consistent with previous reports (3, 27), we found that recombinant FGF21 induced glucose uptake in a dose-dependent manner in 3T3-L1 adipocytes (Fig. 1A). Incubation of cells with 15 μg/ml FGF21 for 24 h increased glucose uptake by 3.5-fold, a magnitude equivalent to that of insulin (Fig. 1B). Furthermore, an additive effect between FGF21 and insulin on stimulation of glucose uptake was observed, confirming that these two hormones act through different pathways in adipocytes. Quantitative real-time PCR analysis showed that FGF21 increased the expression of GLUT1 but had little effect on GLUT4 (Fig. 1C). FGF21-stimulated elevation of GLUT1 mRNA expression was completely abolished by pretreatment of cells with the transcription inhibitor actinomycin D (Fig. 1D), suggesting that FGF21 induces GLUT1 gene through transcriptional activation instead of mRNA stabilization at the posttranscriptional level.

In adipocytes, FGF21 has been shown to activate several protein kinase cascades, including ERK1/2 MAP kinase (3, 9, 19), protein kinase B/Akt (9, 20), and AMP-activated protein kinase (14). We next investigated which of these protein kinases were involved in FGF21-induced GLUT1 expression and glucose uptake in 3T3-L1 adipocytes. Treatment of 3T3-L1 adipocytes with 2 μg/ml FGF21 induced the activation of both ERK1/2 and Akt, as determined by Western blot analysis for phosphorylation at their activation sites (Fig. 2, A and B). However, we did not observe any change in phosphorylation of AMPK at its activation site Thr172 (Fig. 2C). FGF21-induced up-regulation of GLUT1 and elevation of glucose uptake were markedly attenuated by the ERK1/2 specific inhibitor PD98059 (25 μM) (Fig. 2, D and E) but not the Akt inhibitor AktI-1 (Fig. 2F). In addition, the AMPK inhibitor compound C had little effect on FGF21-stimulated GLUT1 expression and glucose uptake (data not shown). Taken in conjunction, these findings suggest that ERK1/2 mediates the transcriptional activation of the GLUT1 gene by FGF21.

FGF21 Transactivates the GLUT1 Gene through the Putative ETS and SRE Recognition Sites Located in the Enhancer Region of the Gene—To investigate how GLUT1 is transactivated by FGF21, we constructed several luciferase reporter vectors driven by the 3.7, 2.7, and 1.7 kb of the mouse GLUT1 promoter, respectively (Fig. 3A). 3T3-L1 adipocytes were transfected with each of these reporter vectors followed by stimulation with various concentrations of FGF21 to monitor its effect on the promoter activity of GLUT1. This analysis demonstrated that the luciferase activity driven by the 3.7-kb GLUT1 promoter was elevated by ~3-fold upon treatment with 2 μg/ml FGF21 for 24 h (Fig. 3E). The basal activity driven by the 3.7-kb GLUT1 promoter was abolished by pretreatment of cells with the transcription inhibitor actinomycin D (Fig. 3D), suggesting that FGF21 induces GLUT1 gene through transcriptional activation instead of mRNA stabilization at the posttranscriptional level.

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3B), whereas the response of the luciferase reporter vector containing both the 2.7- and 1.7-kb promoter was completely abolished (Fig. 3B), suggesting that the FGF21-responsive DNA elements are located within the −3.7 kb and −2.7-kb region. To identify the minimal cis-DNA element(s) that mediates the transactivation of GLUT1 by FGF21, we generated a panel of luciferase reporter vectors that contain progressively truncated GLUT1 promoter spanning from 3.7 to 2.8 kb of the gene and evaluated their response to FGF21 in 3T3-L1 adipocytes (Fig. 3C). The luciferase assay result showed that a 40 bp DNA fragment spanning −3145 bp to −3105 bp of the GLUT1 promoter was indispensable for FGF21 caused gene transcription (Fig. 3D).

We next analyzed potential transcription factor binding sites within this promoter region using both Mat Inspector (genomatix) and TF search programs (TFSEARCH). This bioinformatic analysis identified two adjacent putative DNA binding motifs that were identical to the consensus recognition sequences of ETS (CAGG(A/G)2) and SRE (CC(T/A)6) (Fig. 4A). DNA sequence alignment analysis demonstrated that these two recognition sites were highly conserved among several mammalian species examined. Noticeably, both ETS and SRE are responsive to ERK1/2 (28).

To investigate whether the two putative SRE and ETS recognition sites were indeed critical cis-elements responsible for FGF21-induced transactivation of the GLUT1 gene, we generated two mutant luciferase reporter vectors, 3.7kbmu-luc (SRE) and 3.7kbmu-luc (ETS), in which two nucleotides within each of the consensus binding motifs were mutated (Fig. 4B). In 3T3-L1 adipocytes, the luciferase activities driven by either 3.7kbmu-luc (SRE) or 3.7kbmu-luc (ETS) were slightly decreased compared to that driven by the wild type promoter under the basal condition (Fig. 4C). However, mutation in either one of these two putative SRE and ETS cis-elements completely abolished FGF21-induced increase in the luciferase activity, suggesting that both the putative SRE and ETS recog-
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**FIGURE 4.** The putative SRE and ETS-binding sites within −3145 and −3105 of the promoter confer FGF21-induced transactivation of the GLUT1 gene. A, multiple sequence alignment of −3145 to −3105 bp of the GLUT1 promoter region from four mammalian species is shown. The DNA sequences inside the boxes are identical to the consensus SRE and ETS binding motifs respectively. B, shown is a schematic presentation of the luciferase reporter vectors driven by a 3.7-kb wild type GLUT1 promoter (3.7kb-luc) or mutant GLUT1 promoters bearing mutations within either SRE (3.7kbmu-luc(SRE)) or ETS-binding motif (3.7kmu-luc(ETS)). The mutated nucleotides within each recognition motif are highlighted in bold. C, promoter activities of wild type and mutant promoter regions either at the basal or FGF21-stimulated condition are shown. ***, p < 0.01; ***, p < 0.001 (n = 4).

**FIGURE 5.** FGF21 induces the recruitment of endogenous Elk-1 and SRF to the GLUT1 gene promoter through ERK1/2. A ChIP assay was performed as described under “Experimental Procedures” to quantify the interaction between endogenous GLUT1 promoter with Elk-1 and SRF, respectively. 3T3-L1 adipocytes were preincubated without or with the ERK1/2 inhibitor PD98059 (PD, 25 μM) for 30 min and then treated with 2 μg/ml FGF21 for various periods. Chromatin was isolated and subjected to immunoprecipitation using antibodies specific to Elk-1 or SRF or non-immune rabbit IgG as a negative control. The relative abundance of the GLUT1 promoter spanning −3182 and −3041 bp was quantified by real time PCR and expressed as fold of untreated control. **, p < 0.05; ***, p < 0.01 versus the group precipitated with non-immune IgG (n = 4).
cant reduction in FGF21-induced glucose uptake and phosphorylation of ERK1/2 (Fig. 7, B and E). Additionally, FGF21-induced transcriptional activation of the GLUT1 gene, as determined by both the luciferase reporter assay and quantitative real-time PCR, was markedly blunted upon knocking down of klotho expression (Fig. 7, C and D).

FGF21-induced Activation and Recruitment of Elk-1 and SRF to the GLUT1 Promoter Is Impaired in Obese Mice—FGF21 levels are elevated in obese ob/ob and db/db mice and positively correlate with adiposity in humans (30). Emerging evidence suggests the existence of “FGF21 resistance” in obesity (31), a phenomenon reminiscent of insulin resistance. To confirm the pathophysiological relevance of our findings above, we compared the effects of FGF21-induced signaling pathways leading to GLUT1 expression in adipose tissue collected from lean mice and dietary obese mice. Upon feeding with high fat diet for 12 weeks, mice developed obvious obesity, glucose tolerance and insulin resistance compared with age-matched lean controls (data not shown). In epididymal fat pads isolated from lean mice, a single intravenous injection with FGF21 increased phosphorylation of both Elk-1 and SRF (Fig. 8, A and B) and also induced a time-dependent association of both Elk-1 and SRF with the endogenous promoter, as determined by ChIP analysis (Fig. 8C). Compared with lean mice, FGF21-induced phosphorylation of Elk-1 and SRF and the association of Elk-1 and SRF to the GLUT1 promoter in epididymal fats of diet-induced obese mice were significantly blunted. Likewise, single intravenous injection of FGF21 resulted in a marked elevation of the GLUT1 gene expression in epididymal fats of lean mice, but such an effect of FGF21 was diminished in diet-induced obese mice (Fig. 8D). Taken together, these findings suggest that impaired activation of SRF and Elk-1 may be attributed to FGF21 resistance in obesity.

DISCUSSION

FGF21 has emerged as an important metabolic regulator with multiple salutary effects on glucose and lipid metabolism in animal models. In particular, administration of recombinant FGF21 decreases blood glucose in both diabetic mice and rhesus monkeys (3, 8, 32, 33). A growing body of evidence suggests that the glucose-lowering effect of FGF21 is attributed in part to its ability in promoting glucose uptake in adipocytes through an insulin-independent mechanism (3, 27). However, the precise signaling events that confer the effect of FGF21 on glucose uptake remain elusive. In the present study we showed that FGF21 enhances glucose uptake by transcriptional activation of the GLUT1 gene. Furthermore, we have identified two ERK1/2-responsive transcription factors (Elk-1 and SRF) that mediate FGF21-induced GLUT1 expression by binding to a highly conserved ETS and SRE cis-element located between −3128 and −3105 of the GLUT1 gene promoter.
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The classical members of FGF family exert their biological actions by binding to FGF tyrosine kinase receptors, which include seven different isoforms (34). Most FGF molecules possess a strong heparin-binding property and are capable of binding to FGF receptors with high affinity. However, FGF21 belongs to the unique FGF19 subfamily that exhibits little activity to bind heparin, which enables it to escape from the extracellular space into the circulation (29). However, similar to FGF23, FGF21 requires a specific cofactor for its binding to a certain type of FGF21 receptor and subsequent activation of FGF21 signaling pathways. Several in vitro studies have demonstrated β-klotho as a candidate coreceptor essential for bioactivities of FGF21 (21, 22) through its interaction with the carboxyl terminus of FGF21 (35). Unlike the ubiquitous expression pattern of FGF21 receptors, β-klotho expression is restricted to a number of tissues (adipose tissue, liver, and pancreas), which may explain the tissue selectivity of FGF21 targets. In line with these findings, our present study demonstrated an indispensable role of β-klotho in FGF21-induced ERK1/2 activation, GLUT1 gene transactivation, and glucose uptake in adipocytes. In contrast, a recent study on β-klotho knock-out mice suggests that it is not required for FGF21 signaling pathways leading to the expression of hormone sensitive lipase and Atgl (23). However, whether or not β-klotho is essential for FGF21-induced GLUT1 expression and glucose uptake has not been addressed in this study.

Another notable observation of this study is the additive effects of FGF21 and insulin on glucose uptake in adipocytes, further supporting the notion that these two metabolic hormones modulate glucose uptake through distinct signaling pathways. Interestingly, insulin-stimulated glucose uptake is rapid and transient (36), whereas FGF21 induces a slow, but sustained increase in glucose uptake (3), suggesting that these two hormones may be functionally complementary. Insulin increases glucose uptake by promoting the plasma translocation of GLUT4, which is mediated by PI3K/Akt signaling pathway. Although our present study also observed a modest activation of Akt upon FGF21 stimulation, pharmacological inhibition of either Akt or PI3K has no obvious effect on FGF21-induced GLUT1 expression and glucose uptake. Instead, pharmacological inhibition of ERK1/2 by PD98059 results in a complete abrogation of FGF21-stimulated transactivation of GLUT1 gene and glucose uptake. This finding is consistent with the previous reports that ERK1/2 is the major kinase conferring the metabolic actions of FGF21 in adipocytes (3, 9). In a recent report by Chau and co-workers (14), chronic treatment of adipocytes with FGF21 activates AMPK and SirT1, which in turn enhances mitochondria oxidative capacity. It is well established that AMPK activation promotes glucose uptake by facilitating both transcription and plasma membrane translocation of GLUT4 in an insulin-independent manner (37, 38). Interestingly, the pharmacological activator of AMPK, aminooimidazole carboxamide ribonucleotide, stimulates 2-deoxyglucose uptake in skeletal muscle from both healthy and diabetic people, which was positively correlated with ERK1/2 phosphorylation (20, 29). It is noteworthy that the increase in ERK1/2 phosphorylation during aminooimidazole carboxamide ribonucleotide infusion is insulin-independent as evidenced by a lack of change in either the circulating insulin concentration or the phosphorylation of Akt. However, the results from the present study failed to detect any effect of FGF21 on AMPK activation within 24 h after treatment. Furthermore, the AMPK pharmacological inhibitor does not affect FGF21-induced GLUT1 expression and glucose uptake.

Although up-regulation of GLUT1 by FGF21 has been reported previously (3), the transcriptional events underlying this FGF21 action remain unclear. In the present study we provided several pieces of evidence demonstrating that the transcription factors Elk-1 and SRF act in concert to mediate FGF21-induced transactivation of the GLUT1 gene. First, the cis-element that confers FGF21-induced transactivation of the GLUT1 gene is the consensus binding site for both transcription factors. Second, in response to FGF21 stimulation, both transcription factors are recruited to the GLUT1 gene promoter. Third, FGF21-induced activation of the GLUT1 gene promoter, GLUT1 gene expression, and glucose uptake are all inhibited by siRNA-mediated knockdown of either Elk-1 or

The findings of this study suggest that GLUT1 gene transcription, and glucose uptake in adipocytes are mediated in a complex manner through the activation of Elk-1, SRF, and AMPK, which together promote GLUT1 gene expression and glucose uptake.
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Despite the multiple beneficial effects of FGF21 on glucose and lipid metabolism, its circulating levels are elevated in obese individuals and patients with obesity-related disorders, including metabolic syndrome, diabetes, and nonalcoholic fatty liver disease (30, 31, 45), suggesting the presence of “hyper-FGF21-nemia” that is reminiscent of the hyperinsulinemia in obese and insulin-resistant status. Indeed, FGF21 resistance has been observed in obese animals (31). In db/db obese mice with frank diabetes, the glucose-lowering effect of FGF21 is significantly attenuated when compared with that in lean littermates (30). Furthermore, diet-induced obese mice exhibit a marked attenuation in FGF21-induced activation of ERK1/2 and induction of c-fos and EGR1 in both adipose tissue and fat (31). Consistently, our present study demonstrated that the FGF21-induced signaling pathway leading to GLUT1 gene transactivation is blunted in adipose tissue of obese mice, indicating that this impairment may explain in part the reduced glucose-lowering effect of FGF21 in obese mice. Taken in conjunction, these findings suggest that FGF21 resistance and insulin resistance co-exist and may act in parallel contributing to the pathogenesis of obesity-related disorders such as type 2 diabetes.

In summary, the present study demonstrates that FGF21-induced GLUT1 expression is mediated by β-klotho-ERK1/2-Elk-1/ETS signaling cascade, which in turn transactivates the GLUT1 gene through a highly conserved cis-element within its promoter (Fig. 9). The impairment in this signaling pathway may contribute to FGF21 resistance. Further detailed studies on the molecular mechanism whereby obesity impairs this signaling pathway may provide useful information for developing FGF21-based therapy against obesity-related metabolic disorders.

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