Circulating FGF21 is Liver Derived and Enhances Glucose Uptake During Refeeding and Overfeeding

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ABSTRACT

Fibroblast growth factor 21 (FGF21) is an endocrine hormone that is expressed in multiple tissues and functions physiologically to maintain energy homeostasis. FGF21 is being pursued as a therapeutic target for diabetes and obesity because of its rapid and potent effects on improving insulin sensitivity. However, whether FGF21 enhances insulin sensitivity under physiologic conditions remains unclear. Here we show that liver-derived FGF21 enters the circulation during fasting, but also remains present and functional during the early stage of refeeding. Following a prolonged fast, FGF21 acts as an insulin sensitizer to overcome the peripheral insulin resistance induced by fasting, thereby maximizing glucose uptake. Likewise, FGF21 is produced from the liver during overfeeding and mitigates peripheral insulin resistance. Diet-induced obese FGF21 liver-specific knockout, but not FGF21 adipose-specific knockout, mice have increased insulin resistance and decreased brown adipose tissue mediated glucose disposal. These data are compatible with the concept that FGF21 functions physiologically as an insulin sensitizer under conditions of acute refeeding and overfeeding.
INTRODUCTION

Fibroblast growth factor 21 (FGF21) is an endocrine hormone that signals through a cell-surface receptor complex composed of a classic FGF receptor, FGFR1c, and the FGF co-receptor, β-klotho [1]. FGF21 is expressed in several tissues including liver, white adipose tissue (WAT), brown adipose tissue (BAT) and pancreas and has multiple proposed physiological functions [2], some of which remain controversial [3]. Pharmacologically, FGF21 is a potent insulin sensitizer that improves metabolic dysfunction in a number of obese animal models and humans [4, 5]. A single pharmacological dose of FGF21 to obese mice can decrease plasma glucose levels by ~40-50 percent within 1 hour, an effect lasting up to 6 hours [6]. Additionally, extended administration of FGF21 to obese rodents and primates significantly increases energy expenditure and weight loss [7-9]. Although incompletely understood, adipose tissue is particularly important for the pharmacological actions of FGF21 as the hormone’s acute glucose lowering effect is lost in mice lacking either β-klotho [10] or FGFR1c in adipose tissue [11]. Here we show that circulating FGF21 levels are completely derived from the liver during prolonged fasting and diet induced obesity. Interestingly, loss of FGF21 in the liver, but not adipose tissue, impairs insulin mediated glucose uptake during refeeding and overfeeding. These data show that FGF21 functions as an insulin sensitizer under specific physiologic conditions.
RESEARCH DESIGN AND METHODS

Animals. FGF21 knockout [12], albumin-Cre [13], adiponectin-Cre [14, 15], and FLP-transgenic mice [16] have been described. FGF21^[fl/fl] mice were generated by crossing the FGF21^[neo-loxP/+] mice [12] with FLP-transgenic mice to remove the neo cassette. Subsequent FGF21^[fl/+ ] mice were backcrossed 7 generations to C57Bl/6 mice. FGF21^[fl/+ ] mice were then crossed together to generate FGF21^[fl/fl] mice. FGF21^[fl/fl] mice were then crossed to albumin-Cre or adiponectin-Cre transgenic mice (C57Bl/6J background) to generate liver-specific FGF21 knockout (FGF21^[fl/fl;Albumin-Cre]) or adipose-specific FGF21 knockout (FGF21^[fl/fl;Adiponectin-Cre]) mice, respectively. Mice were maintained on chow (2920X (Harlan Teklad)) or high fat diet (HFD; Research Diets (D12492i)). Twenty-four hour fasting experiments were performed from 9 am to 9 am and refeeding was performed via oral gavage as described [17]. Plasma glucose, insulin, triglycerides, NEFAs, and β-hydroxybutyrate were determined as described [12, 17]. To measure plasma glucagon, plasma was mixed with aprotinin and then snap-frozen in liquid nitrogen and stored at -80°C until assayed by the Vanderbilt Hormone Assay Core. Plasma FGF21 was measured using an FGF21 ELISA assay (Biovendor). All procedures and use of mice were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

In Vivo Glucose Uptake Assays. 6 week old male WT and FGF21 liver-specific knockout mice were placed on a HFD for 6 weeks. Following an overnight fast, tail blood was collected from all mice (time=0). All mice were then injected intraperitoneally with 8-10µCi of [3H]-2-deoxyglucose in a 20% glucose solution and tail blood was collected over the course of 60 min (15 min, 30 min, and 60 min). At the end of the time course all mice were sacrificed by decapitation and tissues immediately dissected, flash frozen in liquid nitrogen and placed at -
80°C until analysis. Plasma radioactivity [18] and determination of tissue-specific uptake of
\[^{3}\text{H}\]-2-deoxyglucose was performed as described [19, 20].

**Isolation of primary brown and white adipocytes.** Primary brown and white preadipocytes
were isolated from the intrascapular brown fat and white fat depots, respectively, of 4-day old
C57Bl6 pups as described [21].

**In Vitro Glucose Uptake.** Primary adipocytes were treated with one of the following conditions
prepared in glucose free media: vehicle, insulin (100nM), FGF21 (1µg/ml) or insulin combined
with FGF21. Recombinant FGF21 generation and purification have been described previously
[12]. Adipocytes were treated for 60 minutes, followed by in vitro glucose uptake assays
performed as described [7].

**Data Analysis.** Gene expression analyses were performed as described [12]. Statistical
comparison of two groups was determined using Student’s t-test.
RESULTS AND DISCUSSION

**Plasma FGF21 levels are derived from the liver and regulate fasting and refeeding responses.** To examine the physiological conditions regulating FGF21 levels, we profiled plasma FGF21 protein levels during fed, fasted or refeed conditions and compared them with circulating insulin levels. Plasma FGF21 levels were increased with 24 hour fasting and remained elevated 15 minutes after refeeding (Supplementary Fig. 1A), when insulin levels were also elevated (Supplementary Fig. 1B). The increased circulating FGF21 concentrations observed after refeeding are likely residual levels produced during fasting, as circulating FGF21 levels return to those of the fed state by 1 hour (Supplementary Fig. 1A) consistent with the half-life of FGF21 in mice being less than 30 minutes [9]. Notably, the time period immediately after refeeding is a unique physiological condition in which both FGF21 and insulin are circulating.

To determine which tissues contribute to circulating FGF21, we generated liver-specific FGF21 knockout mice (FGF21 LivKO) by crossing FGF21\textsuperscript{fl/fl} mice with albumin-Cre transgenic mice. Fed, fasted, or refeed WT and FGF21 LivKO mice were analyzed. While Fgf21 mRNA was induced in the livers of fasted and refeed WT mice, Fgf21 mRNA expression was completely abolished in the livers (Fig. 1A), but not epididymal white adipose tissue (eWAT) (Fig. 1B), of FGF21 LivKO mice. Plasma FGF21 was induced during fasting and refeeding in WT mice, but was abolished in FGF21 LivKO mice (Fig. 1C). There was no difference in the level of fed plasma glucose between groups. However, fasted FGF21 LivKO mice exhibited a slight, but significant, decrease in plasma glucose (Fig. 1D). Importantly, refeed FGF21 LivKO mice had significantly elevated plasma glucose compared to WT littermates (Fig. 1D) despite having similar plasma insulin (Fig. 1E) and adiponectin levels (Fig. 1F). FGF21 LivKO mice displayed an elevation in their glucose excursion curves compared to WT littermates when subjected to a
glucose tolerance test (GTT) (Fig. 1G), despite having insulin levels similar to control (data not shown). Together, these data show that the function of FGF21 extends beyond fasting into the early refeeding response to enhance insulin stimulated glucose uptake.

Liver, but not adipose, produces circulating FGF21 to improve insulin sensitivity in diet induced obese mice. Circulating FGF21 levels are elevated in obese and diabetic rodents and humans (reviewed in [2]). To determine the relative tissue-specific contribution and function of FGF21 during obesity, we generated both liver-specific and adipose-specific diet induced obese (DIO) FGF21 knockout mice. Adipose-specific FGF21 knockout mice (FGF21 AdipoKO) were generated by crossing FGF21<sup>fl/fl</sup> mice with adiponectin-Cre transgenic mice. We then examined high fat diet (HFD) fed diet induced obese (DIO) WT, FGF21 total knockout, FGF21<sup>fl/fl</sup>, FGF21 LivKO, and FGF21 AdipoKO mice. Fgf21 mRNA expression was completely abolished in the livers of DIO FGF21 LivKO (Fig. 2A), while eWAT (Fig. 2B) and BAT Fgf21<sup>fl/fl</sup> mRNA levels remained unchanged (Fig. 2C). In contrast, the levels of Fgf21 mRNA were significantly reduced in eWAT (Fig. 2B) and BAT (Fig. 2C) of DIO FGF21 AdipoKO mice, while levels of hepatic Fgf21 mRNA expression remained unchanged (Fig. 2A). Notably, plasma FGF21 levels were completely abolished in DIO FGF21 LivKO mice, while DIO FGF21 AdipoKO mice had normal levels of plasma FGF21 (Fig. 2D). These results demonstrate that elevated circulating FGF21 in obesity is derived from the liver.

To assess the function of hepatic and adipose FGF21, FGF21 LivKO and FGF21 AdipoKO mice were phenotypically characterized on normal chow and a HFD. Body weight (Supplementary Fig. 2A,B), percent fat (Supplementary Fig. 2C,D), and percent lean mass (Supplementary Fig. 2E,F) were not statistically different between FGF21 LivKO, FGF21
AdipoKO mice and their age and sex matched control littermates (FGF21\textsuperscript{fl/fl}) either at baseline or on HFD. However, during HFD feeding FGF21 AdipoKO mice exhibited a trend towards reduced percent fat at the 8 week ($P = 0.055$) and 10 week ($P = 0.086$) time points (Supplementary Fig. 2D). No statistically significant difference in food intake, plasma triglycerides, non-esterified free fatty acids (NEFAs), glucagon and ketones was observed between DIO WT and FGF21 LivKO or DIO WT and FGF21 AdipoKO mice (Table 1).

To determine whether DIO FGF21 LivKO or FGF21 AdipoKO mice had impaired glucose homeostasis, mice were subjected to glucose and insulin tolerance tests (GTTs and ITTs, respectively). Unlike lean mice, fasted DIO WT mice (3.0 $\pm$ 0.50 ng ml$^{-1}$) show similar circulating FGF21 levels as fed DIO WT mice (2.8 $\pm$ 0.20 ng ml$^{-1}$). DIO FGF21 LivKO mice, but not DIO FGF21 AdipoKO mice, exhibited a markedly increased glucose excursion curve in response to the GTT compared to WT littermates (Fig. 2E,F). DIO FGF21 LivKO mice also possessed significantly elevated plasma insulin levels throughout the glucose tolerance test, suggesting increased insulin resistance (Fig. 2G). Insulin tolerance tests revealed that DIO FGF21 LivKO mice had an impaired response to insulin compared to DIO wild-type littermates (Fig. 2I), while DIO FGF21 AdipoKO mice had normal insulin tolerance (Fig. 2J). Consistent with increased insulin resistance, DIO FGF21 LivKO mice displayed increased hepatic lipid accumulation (Fig. 2K and Supplementary Figure 2G) and white adipose tissue fibrosis compared to WT littermates (Fig. 2G), an effect not observed between DIO FGF21 AdipoKO and WT littermates (Fig. 2K). Recently, it was reported that FGF21 functions pharmacologically by inducing adiponectin levels [22, 23]. Plasma adiponectin levels were not significantly different in FGF21 LivKO or FGF21 AdipoKO mice compared to WT controls (Table 1).
Therefore, loss of hepatic, but not adipose, FGF21 levels impairs insulin sensitivity in HFD fed mice independent of changes in adiponectin levels.

**FGF21 is induced in liver by HFD feeding to drive glucose disposal into BAT in vivo.**

To determine which tissue(s) have reduced glucose disposal and may be responsible for the DIO FGF21 LivKO phenotype, we analyzed tissue-specific glucose uptake in WT and FGF21 LivKO mice fed a HFD for 6 weeks. This was accomplished by injecting mice with labeled [³H]2-deoxyglucose in a 20% glucose bolus to assess glucose uptake during a physiological insulin response. FGF21 LivKO mice on HFD for 6 weeks also exhibited an increased glucose excursion curve compared to WT controls (Fig. 3A). Notably, this increased glucose excursion in the FGF21 LivKO mice fed HFD for 6 weeks occurred despite having normal plasma insulin levels (Fig. 3B), unlike FGF21 LivKO mice fed HFD for 10 weeks (Fig. 2G). Interestingly, FGF21 LivKO mice had a lower rate of glucose uptake in BAT, but not in eWAT, subcutaneous WAT (scWAT), skeletal muscle or heart (Fig. 3C). We conclude that FGF21 regulates glycemia at least in part by effecting glucose disposal into BAT in DIO mice. FGF21 has also been shown to reduce hepatic glucose production during obesity (reviewed in [2]), so multiple mechanisms may be responsible for FGF21-mediated effects on glucose homeostasis. FGF21 enhances glucose disposal under specific physiologic conditions and a limitation of this study is that only glucose metabolism was examined. Thus, we cannot rule out that other metabolic pathways are regulated by FGF21 under these or other physiological conditions.

We next examined whether FGF21 can act directly on brown adipocytes to stimulate glucose uptake. Primary brown and white adipocytes were isolated and treated with vehicle, insulin, FGF21, or both for 1 hour. While extended treatment (24 hours) of white adipocytes with
FGF21 stimulates glucose uptake independent of insulin [7], 1 hour treatment with FGF21 alone did not stimulate glucose uptake in either brown (Fig. 3D) or white adipocytes (Fig. 3E). Insulin alone, however, was able to significantly increase glucose uptake in brown (Fig. 3D) and white adipocytes (Fig. 3E). Interestingly, co-treatment of insulin with FGF21 synergistically increased glucose uptake in primary brown adipocytes (Fig. 3D), but not white adipocytes (Fig. 3E). These data demonstrate that FGF21 can signal directly to brown adipocytes to enhance insulin-stimulated glucose uptake, and that loss of circulating FGF21 impairs glucose uptake in BAT of DIO mice. These results, however, do not exclude the possibility that FGF21 may also regulate BAT activity through indirect mechanisms (e.g., sympathetic nerve activity) in vivo. Therefore, just as FGF21 functions early during refeeding to mitigate physiological peripheral insulin resistance, FGF21 may also function during overfeeding to overcome diet-induced insulin resistance.

In summary, our data are compatible with FGF21 functioning beyond the fasting/starvation response to enhance insulin action during refeeding and overfeeding. Thus, FGF21 acts as an insulin sensitizer under physiologic conditions which may explain its acute pharmacological actions. FGF21 may promote glucose uptake into adipose during refeeding to maximize energy replenishment and during overfeeding to protect against lipotoxicity. Interestingly, the ability of FGF21 to enhance insulin action may explain why pharmacological administration of FGF21 increases insulin sensitivity in obese mice, where circulating insulin levels are already elevated, but not in lean mice. Understanding the mechanisms regulating FGF21-mediated enhancement of insulin sensitivity may provide important insight into new treatments for metabolic disease.
ACKNOWLEDGMENTS

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K.R.M. and M.J.P. designed the experiments. K.R.M. and M.C.N performed in vitro and in vivo radioactive glucose uptake assays. M.C.N., M.K.A, and M.D.A. performed gene expression analyses. D.J.M., S.A.K., and M.M. generated critical reagents. K.R.M. and M.J.P. wrote the manuscript and M.C.N., M.K.A., M.D.A., D.J.M., S.A.K., and M.M. reviewed the manuscript. M.J.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors have no conflicts to declare.
REFERENCES


Table 1. Plasma parameters of diet-induced obese (DIO) FGF21 tissue-specific knockout mice.

<table>
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<th>DIO FGF21&lt;sup&gt;fl/fl&lt;/sup&gt;</th>
<th>DIO FGF21 LivKO</th>
<th>DIO FGF21&lt;sup&gt;fl/fl&lt;/sup&gt;</th>
<th>DIO FGF21 AdipoKO</th>
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<tr>
<td>Food intake (g/day)</td>
<td>2.61 ± 0.08</td>
<td>2.60 ± 0.07</td>
<td>2.65 ± 0.06</td>
<td>2.68 ± 0.10</td>
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<td>Triglycerides (mg/dl)</td>
<td>65.70 ± 3.75</td>
<td>62.73 ± 2.70</td>
<td>74.80 ± 4.64</td>
<td>82.58 ± 5.89</td>
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<td>NEFAs (mM)</td>
<td>0.49 ± 0.03</td>
<td>0.50 ± 0.03</td>
<td>0.58 ± 0.04</td>
<td>0.53 ± 0.03</td>
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<tr>
<td>ß-hydroxybutyrate (µM)</td>
<td>54.86 ± 9.87</td>
<td>60.78 ± 6.67</td>
<td>56.67 ± 7.11</td>
<td>43.84 ± 5.09</td>
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<tr>
<td>Glucagon (pg/ml)</td>
<td>85.99 ± 20.26</td>
<td>77.95 ± 26.84</td>
<td>50.78 ± 4.10</td>
<td>65.24 ± 8.56</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>15.07 ± 0.44</td>
<td>14.57 ± 0.17</td>
<td>15.03 ± 0.51</td>
<td>15.27 ± 0.50</td>
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Plasma parameters were measured in diet-induced obese FGF21 liver-specific knockout (FGF21 LivKO), FGF21 adipose-specific knockout (FGF21 AdipoKO) and FGF21<sup>fl/fl</sup> littermate controls. Data are presented as mean ± SEM.
FIGURE LEGENDS

Figure 1. Generation and characterization of liver-specific FGF21 knockout mice. (A-F) 12-14 week old male wild-type (FGF21\textsuperscript{fl/fl}) and FGF21 liver-specific knockout (FGF21 LivKO) mice were either \textit{ad libitum} fed chow, fasted 24 hours (hr), or fasted 24 hrs then refed for 15 min via oral gavage (n = 5-8/group). Levels of (A) hepatic \textit{Fgf21} mRNA, (B) epididymal white adipose tissue (eWAT) \textit{Fgf21} mRNA, (C) plasma FGF21 protein, (D) plasma glucose, (E) plasma insulin, and (F) plasma adiponectin in the indicated mice are shown. Levels of (G) plasma glucose and area under the curve (AUC) from WT and FGF21 LivKO mice during a glucose tolerance test (n = 5-6/group). Data are presented as mean +/- SEM. Different lowercase letters represent statistical significance between genotypes (a, \(P < 0.05\); b, \(P < 0.01\); d, \(P < 0.001\)) and asterisks indicate significance between control mice in different conditions (\(P < 0.05\)).

Figure 2. Loss of liver-derived FGF21, but not adipose-derived FGF21, impairs glucose homeostasis and insulin sensitivity in diet induced obese mice. (A-D) Diet induced obese (DIO) wild-type (WT), FGF21 total knockout (KO), FGF21\textsuperscript{fl/fl}, FGF21 liver-specific KO (FGF21 LivKO), and FGF21 adipose-specific KO (AdipoKO) mice were fed a high fat diet (HFD) for 16 weeks. Levels of (A) hepatic \textit{Fgf21} mRNA, (B) epididymal white adipose tissue (eWAT) \textit{Fgf21} mRNA, (C) brown adipose tissue (BAT) \textit{Fgf21} mRNA, and (D) plasma FGF21 protein in the indicated mice are shown. (E-K) Age-matched male FGF21\textsuperscript{fl/fl}, FGF21 LivKO, and FGF21 AdipoKO mice were individually caged and maintained on chow diet until 10 weeks of age. At 10 weeks of age, all mice were placed on HFD for 10 weeks (n = 8-9/group). FGF21\textsuperscript{fl/fl} control mice were compared only to their knockout littermates. Levels of (E-F) plasma glucose and (G-H) plasma insulin during a glucose tolerance test (n = 6/group). Glucose tolerance tests were
performed by administering 2 g glucose/kg lean body weight via intraperitoneal (i.p.) injection to mice that had been fasted for 16 hours. (I-J) Plasma glucose levels during an insulin tolerance test (n = 5-6/group). Insulin tolerance tests were performed by administering 0.75U insulin/kg body weight via i.p. injection to mice fasted for 4-6 hours. (K) Oil Red O staining of livers and hematoxylin and eosin staining of epididymal white adipose tissue (eWAT). Data are presented as mean +/- SEM. Different lowercase letters represent statistical significance (a, P < 0.05; b, P < 0.01; c, P < 0.005; d, P < 0.001).

**Figure 3. Loss of hepatic FGF21 impairs glucose uptake into BAT in vivo.**

(A) Plasma glucose AUC, (B) plasma insulin levels, and (C) rates of glucose uptake in unanesthetized male WT and FGF21 LivKO fed HFD for 6 weeks. All mice were administered [3H]2-deoxyglucose after an overnight fast; glucose uptake was measured in brown adipose tissue (BAT), epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (scWAT), heart, and skeletal muscle (tibialis anterior) (n = 5-7/group). Glucose uptake assays in (D) primary brown adipocytes and (E) primary white adipocytes treated with vehicle (Veh), insulin (100 nM), FGF21 (1 µg/ml), or FGF21 + insulin. Experiments were performed in triplicate twice. Data are presented as mean +/- SEM. Different lowercase letters represent statistical significance (a, P < 0.05; b, P < 0.01; d, P < 0.001).
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Figure 1
Diabetes

Figure 3

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Supplementary Figure 1

Levels of (A) plasma FGF21 protein and (B) plasma insulin from 12-14 week old wild-type C57Bl/6 male mice subjected to different feeding paradigms: mice were either *ad libitum* fed chow, fasted 24 hours (hr), or fasted 24 hrs then refed for 15 minutes, 1 hr, 2 hr, or 4 hr via oral gavage (n = 4-5/group). Data are presented as mean +/- SEM. Different lowercase letters represent statistical significance (a, $P<0.05$; c, $P<0.005$; d, $P<0.001$).
Supplementary Figure 2

(A-F) FGF21$^{fl/fl}$, FGF21 LivKO, and FGF21 AdipoKO mice were fed a high fat diet for 10 weeks (n = 8-9/group). FGF21$^{fl/fl}$ control mice were compared only to their knockout littermates.

(A-B) Body weight curves, (C-D) percent body fat, and (E-F) percent lean mass in the indicated mice. Body composition was determined using a Bruker Minispec NMR machine. (G) Liver triglycerides from the indicated mice. Data are presented as mean +/- SEM. Different lowercase letters represent statistical significance (c, $P<0.005$).