Lack of Overt FGF21 Resistance in Two Mouse Models of Obesity and Insulin Resistance

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Circulating levels of fibroblast growth factor 21 (FGF21), a metabolic regulator of glucose, lipid, and energy homeostasis, are elevated in obese diabetic subjects, raising questions about potential FGF21 resistance. Here we report tissue expression changes in FGF21 and its receptor components, and we describe the target-organ and whole-body responses to FGF21 in ob/ob and diet-induced obese (DIO) mice. Plasma FGF21 concentrations were elevated 8- and 16-fold in DIO and ob/ob mice, respectively, paralleling a dramatic increase in hepatic FGF21 mRNA expression. Concurrently, expression levels of βKlotho, FGF receptor (FGFR)-1c, and FGFR2c were markedly down-regulated in the white adipose tissues (WAT) of ob/ob and DIO mice. However, dose-response curves of recombinant human FGF21 (rhFGF21) stimulation of ERK phosphorylation in the liver and WAT were not right shifted in disease models, although the magnitude of induction in ERK phosphorylation was partially attenuated in DIO mice. Whole-body metabolic responses were preserved in ob/ob and DIO mice, with disease models being more sensitive and responsive than lean mice to the glucose-lowering and weight-loss effects of rhFGF21. Endogenous FGF21 levels, although elevated in diseased mice, were below the half-maximal effective concentrations of rhFGF21, suggesting a state of relative deficiency. Hepatic and WAT FGF21 mRNA expression levels declined after rhFGF21 treatment in the absence of the increased expression levels of βKlotho and FGFR. We conclude that overt FGF21 resistance was not evident in the disease models, and increased hepatic FGF21 expression as a result of local metabolic changes is likely a major cause of elevated circulating FGF21 levels. (Endocrinology 153: 69–80, 2012)

Fibroblast growth factor (FGF) 21 is a secreted polypeptide and a member of the distinctive FGF19 subfamily (1). FGF21 acts through a receptor complex consisting of a coreceptor, βKlotho, and a canonical FGF receptor (FGFR) located on the surface of the plasma membrane (1, 2). Upon binding to βKlotho, FGF21 preferentially activates c isoforms of the FGFR (3) and elicits intracellular signaling cascades that include phosphorylation of FGFR substrate-2 and ERK (4, 5).

FGF21 is involved in the regulation of glucose, lipid, and energy metabolism in vivo. Transgenic mice overexpressing FGF21 exhibited stunted growth rate and were resistant to diet-induced obesity and insulin resistance (6). Administration of recombinant FGF21 resulted in striking improvements of the metabolic state in rodents and nonhuman primates (6–8). The improvements included lowered blood glucose, insulin, triglyceride, and cholesterol levels; improved lipoprotein profile; increased glucose tolerance and insulin sensitivity; and reduced hepatosteatosis and obesity. Germ-line deletion of the FGF21 gene in mice, however, resulted in mild metabolic phenotypes. FGF21 knockout mice were viable and fertile and did not show obvious metabolic abnormalities when fed a standard chow diet (9, 10). Nevertheless, the knockout mice were
slightly heavier and had an increase in hepatic fat content when challenged with a ketogenic diet (9). Mice with adenovirus-mediated knockdown of hepatic FGF21 also showed elevated hepatic and plasma triglyceride levels when fed a ketogenic diet (11).

The expression of FGF21 is highly regulated by feeding and by levels of dietary fat and carbohydrates (12–14). Starvation or feeding a high-fat or high carbohydrate diet all induced FGF21 mRNA expression in the liver (12–14). FGF21 has been shown to be a direct target gene of the transcriptional factors peroxisome proliferator-activated receptors (PPAR) (11, 15). Treatment with synthetic PPARα and PPARγ agonists increased FGF21 mRNA expression in the liver and adipocytes, respectively (16).

Over the years, research interest in the clinical relevance of FGF21 to human obesity and type 2 diabetes has substantially increased. Most studies emphasized the changes of circulating FGF21 levels in human pathogenic conditions and the association of FGF21 with metabolic parameters. Circulating FGF21 levels increased after prolonged fasting in normal subjects. The distribution of serum FGF21 was skewed, and the interindividual levels could differ by a thousandfold among healthy subjects (12). In obese and diabetic patients, circulating FGF21 levels were generally elevated, and the median value was about twice that seen in healthy subjects (17, 18). A substantial increase in serum FGF21 levels was reported in patients with nonalcoholic fatty liver disease (19). Although the initial association analysis showed positive correlation of serum FGF21 concentrations with multiple metabolic risk factors (17, 20), a later study that used glucose clamp technique indicated that FGF21 levels were associated strongly with plasma and hepatic triglyceride levels and weakly with insulin sensitivity (20).

The elevated circulating FGF21 levels in obese and diabetic patients appeared dissociated with its expected actions to suppress obesity, type 2 diabetes, and hepatosteatosis. Impaired FGF21 action, a state of FGF21 resistance leading to compensatory FGF21 overproduction, was therefore suspected. Elevated circulating FGF21 levels has also been reported in some animal models. In this study, we investigated the expression of FGF21 and its receptor components in two animal models of obesity and insulin resistance, the leptin-deficient ob/ob mice and the diet-induced obese (DIO) mice. We also examined the target-organ and whole-body responses to FGF21 in ob/ob and DIO mice compared with their respective age-matched lean counterparts.

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**Materials and Methods**

**Reagents and animals**

Recombinant human FGF21 (rhFGF21) was prepared as previously described (21). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Amgen Inc. Male ob/ob and C57BL/6 mice were ordered from Harlan and fed a standard rodent chow diet (catalog no. 2020X; Harlan Lab Diets, Madison, WI). To prepare DIO mice, 4-wk-old C57BL/6 male mice were fed a high-fat diet (D12492; Research Diets, Inc., New Brunswick, NJ) for 12 wk. DIO mice were continued on the high-fat diet until study completion.

**FGF21 ELISA**

Concentrations of rhFGF21 in mouse plasma samples were measured using an ELISA developed in-house as described previously (21). The assay showed no cross-reactivity with mouse FGF21.

The ELISA used to determine mouse FGF21 concentrations is as follows: a rat antimouse FGF21 monoclonal antibody and a rabbit anti-FGF21 polyclonal antibody were generated internally and used as capture and detection reagents, respectively. The capture antibody was verified to react specifically with mouse FGF21, with minimal (<1%) cross-reactivity to human FGF21 or FGF19. A 96-well plate was coated with the monoclonal capture antibody specific for mouse FGF21. Standards, quality controls, matrix blank, and unknown samples were loaded into the wells after pretreatment in assay buffer. After a 2-h incubation followed by washing, a biotin-conjugated rabbit anti-FGF21 polyclonal antibody was added. An additional wash step, a streptavidin-polyhorseradish peroxidase conjugate was added. After a final wash, a high-sensitivity luminescent peroxidase substrate was added and a luminescent signal was produced. The conversion of relative luminescent units to concentration for the unknown samples was achieved through a software-mediated comparison with a standard curve assayed on the same plate.

**In vivo FGF21 signaling study**

*Ad libitum*-fed mice were ip injected with vehicle or rhFGF21 at doses of 0.001, 0.01, 0.1, 1, and 10 mg/kg. Mice were placed in a new cage devoid of food after injection. Fifteen minutes later, mice were euthanized by decapitation. Liver and epidydimal white adipose tissues (WAT) were collected and snap frozen in liquid nitrogen for later analysis. In a time-course study, mice were injected with rhFGF21 at 1 mg/kg. WAT were collected from mice at 0, 5, 15, 30, 60, or 120 min after injection.

**Acute in vivo study**

*Ad libitum*-fed mice were ip injected with vehicle or 1 mg/kg rhFGF21. Blood samples (~20 μl) were serially collected from the retroorbital sinus vein of each mouse before injection (0 h) and 2 and 4 h after injection. Blood glucose was measured with a One Touch Ultra glucometer (LifeScan, Inc., Milpitas, CA). The remaining blood samples were then collected into an EDTA tube for the preparation of plasma. Plasma levels of insulin, amylin, and glucagon were measured using a mouse endocrine multiplex assay (Linco Research, St. Charles, MO).

**Subchronic in vivo study**

Eight-week-old lean and ob/ob mice were ip administered with vehicle or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice a day for 22 d. Blood samples were collected from the retroorbital sinus of each *ad libitum*-fed mouse on study d 7 at
1 h after the morning injection. Blood glucose, insulin, amylin, triglycerides, and cholesterol levels were measured. A glucose tolerance test (GTT) was conducted on study d 15 at 1 h after the morning injection. Mice were overnight fasted before the GTT. Body weight was monitored daily. Mice were euthanized on study d 22. Liver and WAT were collected and frozen in liquid nitrogen for later gene expression analysis.

A similar study was conducted in 16-wk-old lean and DIO mice. Mice were ip administered with vehicle or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice a day for 10 d. Blood samples were collected from ad libitum-fed mice on study d 7 at 1 h after the morning injection. A GTT was conducted on study d 10 at 1 h after the morning injection. Body weight was monitored daily.

Quantitative real-time PCR and Western blot analysis

Total RNA was prepared from the frozen liver and WAT tissues. RT-PCR was carried out with 50 ng of total RNA, 300 nM of primers, and a 140-nM probe in a 20-μl volume in a 384-well plate format, as previously described (8). Cyclophilin or 18S was used as the reference gene. Relative expression was determined by using the standard curve or comparative cycle threshold (Ct) methods (22). Reactions with Ct greater than 34 were determined to be below the limit of detection.

Results

Elevated plasma FGF21 concentrations in DIO and ob/ob mice were in parallel with increased FGF21 mRNA expression in the liver

Plasma FGF21 concentrations were determined in ad libitum-fed, 8-wk-old ob/ob mice, 16-wk-old DIO mice, and 8- and 16-wk-old C57BL6 lean mice. Plasma FGF21 concentrations were similar in 8- and 16-wk-old lean mice (0.18 ± 0.02 and 0.24 ± 0.06 ng/ml, respectively), whereas they were dramatically increased in DIO (1.93 ± 0.26 ng/ml) and ob/ob mice (2.85 ± 0.18 ng/ml) by 8- and 16-fold, respectively, compared with the levels seen in age-matched lean mice (Fig. 1A). The plasma levels of FGF21 in ob/ob mice were also statistically significantly higher than those in DIO mice (Fig. 1A). In C57BL6 lean mice, FGF21 mRNA was expressed in the pancreas, liver, WAT, and brown adipose tissues (BAT) and was expressed minimally in the kidney and muscle (Fig. 1B). A marked increase in FGF21 mRNA expression levels, which paralleled the concentrations of circulating FGF21 levels, was observed in the liver of DIO and ob/ob mice (Fig. 1B). The mRNA expression levels of FGF21 were also slightly increased in the WAT of DIO and ob/ob mice (Fig. 1B). Although the pancreas produced abundant amounts of FGF21 mRNA, the pancreatic expression of FGF21 mRNA was attenuated by about 60 and 80% in DIO and ob/ob mice compared with those in lean mice, respectively (Fig. 1B). There were no clear disease-state related changes in FGF21 mRNA amounts in the BAT, skeletal muscle, or kidney (Fig. 1B).

Expression levels of βKlotho, FGFR1c, and FGFR2c were reduced in the WAT of DIO and ob/ob mice

βKlotho was expressed in the WAT, BAT, liver, and pancreas but was expressed minimally in the muscle of ob/ob mice.
C57BL6 lean mice (Fig. 2A). Relative to the levels seen in lean mice, βKlotho expression levels were reduced by approximately 90% in the WAT and remained unchanged in the liver, BAT, pancreas, and muscle of both DIO and ob/ob mice (Fig. 2A). In parallel with mRNA expression levels, βKlotho protein amounts were reduced by 90 and 75% in the WAT of ob/ob and DIO mice, respectively, whereas they were unaltered in the livers of ob/ob and DIO mice relative to those of lean mice (Fig. 2, F–H).

In lean mice, FGFR1c was highly expressed in the WAT and was moderately expressed in the BAT and muscle (Fig. 2B). FGFR2c was also preferentially expressed in the WAT and BAT relative to other metabolic tissues examined (Fig. 2C). Compared with the levels seen in lean mice, the expression levels of FGFR1c and FGFR2c were substantially reduced in the WAT and were unaltered in other tissues tested in both ob/ob and DIO mice (Fig. 2, B and C). FGFR3c was preferentially expressed in the adipose tissue and muscle. Expression levels of FGFR3c in the liver were

**FIG. 2.** Expression levels of βKlotho, FGFR1c, and FGFR2c were reduced in the WAT of DIO and ob/ob mice. A–E, Relative mRNA expression levels of βKlotho (A), FGFR1c (B), FGFR2c (C), FGFR3c (D), and FGFR4 (E) in metabolic tissues of ad libitum-fed lean (open bars), DIO (gray bars), and ob/ob (black bars) mice. Total RNA were extracted and pooled RNA extracts from eight lean mice (8 and 16 wk old, n = 4 each) and four DIO or ob/ob mice were subjected to RT-PCR analysis. Data were normalized to 18S control gene and are expressed as fold change in FGF21 mRNA levels relative to the level seen in the liver of lean mice, which was arbitrarily set to a value of 1.0. The CT value for βKlotho, FGFR1c, FGFR2c, FGFR3c, and FGFR4 was 29, 32, 30, 32, and 29, respectively, in the liver of lean mice. Results represent mean ± SEM of triplicate RT-PCR analysis. F, Western blot analysis of βKlotho in the liver and WAT of lean, ob/ob, and DIO mice. Tissue lysates were prepared from ad libitum-fed, 8-wk-old lean and ob/ob mice or 16-wk-old lean and DIO mice. Tissue lysate containing 25 μg protein was loaded in each lane (n = 4 per group). β-Actin was a loading control. G and H, Densitometry quantification of the Western blots shown in F. The relative level of βKlotho was obtained by normalizing the intensity of the βKlotho band to that of β-actin and then compared with the value of the lean mice, which was arbitrarily set to a value of 1.0. Data are mean ± SEM of four animals. Open bar, lean mice; black bar, ob/ob mice; gray bar, DIO mice.
slightly elevated (~3 fold) in DIO and ob/ob mice compared with those in lean mice, whereas its expression levels in other tissues did not show consistent disease state-related change (Fig. 2D). FGFR4 was mainly expressed in the liver and muscle, and its expression levels were not altered in ob/ob and DIO mice relative to those in lean mice (Fig. 2E).

**FGF21 signaling cascade through ERK phosphorylation was retained in the liver and WAT of ob/ob mice**

The simultaneous down-regulation of βKlotho, FGFR1c, and FGFR2c in the WAT of ob/ob and DIO mice prompted us to examine whether receptor-mediated FGF21 signaling was attenuated. The ability of rhFGF21 to stimulate ERK phosphorylation in the liver and WAT was determined in age-matched lean and ob/ob mice by conducting dose-response and time-course studies. Fifteen minutes after ip injection of rhFGF21, ERK phosphorylation was dose dependently increased in the liver and WAT of both lean and ob/ob mice (Fig. 3A). Dose-response curves were constructed by expressing the relative phosphorylated ERK (p-ERK)/total ERK value as a function of rhFGF21 dose (Fig. 3B). The relative p-ERK/total ERK value was used because it normalized the difference in the levels of basal p-ERK and total ERK observed between diseased and lean mice and would represent an
FGF21-specific effect. The dose-response curves of ERK phosphorylation derived from the liver and WAT of ob/ob mice were similar to those from the liver and WAT of lean mice (Fig. 3B). There was no rightward shift in the dose-response curves; the ED₅₀ of rhFGF21 was also comparable between lean and ob/ob mice in the liver (0.07 mg/kg vs. 0.01 mg/kg lean vs. ob/ob) and in the WAT (0.004 mg/kg vs. 0.002 mg/kg, lean vs. ob/ob) (Fig. 3B, top panel). The ED₅₀ values in the WAT appeared lower than those in the liver, regardless of whether the mice were lean or ob/ob, suggesting that WAT is more sensitive to FGF21 than the liver is. The maximum induction in ERK phosphorylation was achieved in ob/ob mice in the liver and WAT (Fig. 3B). In a time-course study, the induction in ERK phosphorylation reached the maximum level in less than 5 min and remained elevated up to 60 min in the WAT of both lean and ob/ob mice (Fig. 3, C and D). The magnitude of induction in ERK phosphorylation was similar in lean and ob/ob mice (Fig. 3, C and D).

Lack of whole-body FGF21 resistance in ob/ob mice

The in vivo metabolic responses were compared in lean and ob/ob mice after acute and chronic administration of rhFGF21. Eight-week-old lean and ob/ob mice fed ad libitum were ip injected with rhFGF21 at 1 mg/kg. At 2 h after injection, blood glucose, insulin, and amylin levels in rhFGF21-treated ob/ob mice were reduced by 60, 55, and 50%, respectively, compared with those in vehicle-treated ob/ob mice (Fig. 4, A–C). The effects were sustained for more than 4 h (Fig. 4, A–C). The areas under the curve (AUC) of glucose, insulin, and amylin over the 4-h period were reduced by 46% (P < 0.001), 38% (P < 0.001), and 23% (P < 0.01), respectively, in rhFGF21-treated ob/ob mice compared with vehicle-treated ob/ob mice. Treat-

**FIG. 4.** Acute and subchronic metabolic effects of rhFGF21 in lean and ob/ob mice. A–D, Ad libitum fed 8-week-old lean and ob/ob mice were ip injected with vehicle (closed circles) or 1 mg/kg rhFGF21 (open circles). Blood samples were collected before injection (0 h), 2 and 4 h after injection. Blood glucose (A), plasma levels of insulin (B), amylin (C) and glucagon (D) were measured. Data are means ± SEM, n = 15 per group; *P < 0.05; **P < 0.01; ***P < 0.001 compared with vehicle-treated group at each time point. E–M, Dose-response effects of rhFGF21 on metabolic parameters in lean and ob/ob mice. 8-week-old lean (solid line and closed circle) and ob/ob mice (dashed line and open circle) were ip administered with vehicle (0) or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice daily for 22 d. Blood samples were collected from ad libitum-fed mice on study d 7 at 1 h after the morning injection for measurements of blood glucose (E), plasma insulin (G), triglyceride (H), and cholesterol levels (I). F, A GTT was conducted on study d 15 at 1 h after the morning injection. Glucose AUC were calculated and expressed. J, Percent change in body weight relative to baseline on study d 14. Liver triglycerides (K), liver cholesterol (L), and liver weights (M), were measured from tissues collected on study d 22 at 1 h after the morning injection. Data are means ± SEM (n = 8 per dose group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with vehicle-treated lean or ob/ob mice.
ment of lean mice with rhFGF21 only caused a marginal reduction in glucose, insulin, and amylin levels at either the 2- or 4-h time point (Fig. 4, A–C). The glucose AUC over the 4-h period was reduced by 10% (P < 0.05) in rhFGF21-treated lean mice, whereas the AUC of insulin and amylin did not change compared with those of vehicle-treated lean mice. Treatment with rhFGF21 had no effect on plasma glucagon levels in either lean or ob/ob mice (Fig. 4D).

In a dose-response study conducted in 8-wk-old lean and ob/ob mice, administration of rhFGF21 dose dependently reduced blood glucose levels and improved glucose tolerance in ob/ob mice; the ED50 value was estimated to be 0.01 mg/kg (Fig. 4, E and F). Plasma insulin levels were reduced in ob/ob mice receiving rhFGF21 at a dose as low as 0.001 mg/kg (Fig. 4G). Similar treatment of lean mice with rhFGF21 did not change blood glucose levels or GTT AUC at any doses given (Fig. 4, E and F). Insulin levels showed a trend toward reduction in lean mice receiving rhFGF21 doses of 0.1 mg/kg or greater (Fig. 4G).

Plasma and hepatic triglyceride concentrations were dose dependently reduced in both lean and ob/ob mice (Fig. 4, H and K). Lean mice appeared to be more sensitive to the triglyceride-lowering effect of rhFGF21 than ob/ob mice. Reductions in plasma and hepatic triglyceride levels were observed in lean mice receiving 0.001 mg/kg rhFGF21, whereas the effects were observed in ob/ob mice receiving 0.1 mg/kg or greater rhFGF21 (Fig. 4, H and K). Treatment with rhFGF21 for 7 d did not cause a clear dose-dependent effect on plasma cholesterol levels in either lean or ob/ob mice (Fig. 4I). Liver cholesterol and liver weight were reduced in ob/ob mice receiving rhFGF21 at doses of 0.1 mg/kg or greater, whereas these effects were not observed in lean mice (Fig. 4, L and M). Administration of rhFGF21 produced a similar dose-dependent inhibition in body weight gain in lean and ob/ob mice (Fig. 4J).

**FGF21 mRNA expression declined in the liver and WAT of ob/ob and lean mice after rhFGF21 treatment**

Administration of rhFGF21 dose dependently reduced hepatic and WAT FGF21 mRNA expression levels in lean and ob/ob mice (Fig. 5A). Treatment of lean and ob/ob mice with increasing doses of rhFGF21 for 22 d did not change mRNA expression levels of βKlotho, FGFR1c, FGFR3c, or FGFR4 in either the liver or WAT (Fig. 5, B, C, E, and F). The expression levels of FGFR2c mRNA were, however, substantially reduced in the WAT of lean mice receiving rhFGF21 at a dose as low as 0.001 mg/kg (Fig. 5D). The down-regulation of FGFR2c was not observed in the WAT of ob/ob mice possibly because ob/ob had reduced levels of WAT FGFR2c mRNA relative to the levels seen in lean mice (Fig. 5D, lower panel). FGFR2c mRNA levels were unchanged in the liver of lean or ob/ob mice following rhFGF21 treatment (Fig. 5D, upper panel).

**Plasma rhFGF21 concentrations relative to endogenous FGF21 levels in lean and ob/ob mice**

Plasma concentrations of rhFGF21 were determined in lean and ob/ob mice 15 min after a single ip injection of rhFGF21 at doses of 0.001, 0.01, 0.1, and 1 mg/kg. Plasma concentrations of rhFGF21 increased dose proportionately and were comparable in lean and ob/ob mice at any of the doses given (Fig. 6). The mean concentration of rhFGF21 was 6.7 ± 0.4 and 5.3 ± 0.5 ng/ml in lean and ob/ob mice, respectively, when rhFGF21 was administered at 0.001 mg/kg (Fig. 6). A 10-fold increase in rhFGF21 dose yielded an approximately 10-fold increase in plasma concentrations (Fig. 6). Plasma concentrations of mouse endogenous FGF21 were also determined in the same cohort of lean and ob/ob mice receiving no rhFGF21 injection. Consistent with previous observations (Fig. 1A), plasma concentrations of mouse FGF21 were 0.43 ± 0.03 ng/ml in lean mice and 3.4 ± 0.34 ng/ml in ob/ob mice (Fig. 6). Although elevated, the mouse endogenous FGF21 levels in ob/ob mice were close to plasma concentrations of rhFGF21 given at a dose of 0.001 mg/kg, a starting effective dose observed in vivo; and were below plasma concentrations of rhFGF21 given at doses of 0.01 mg/kg or greater, ED50 doses for glucose, and body weight-lowering (Fig. 6).

**Lack of whole-body FGF21 resistance in DIO mice despite attenuated induction in ERK phosphorylation in the liver and WAT**

The FGF21-induced signaling cascade on ERK phosphorylation and whole-body metabolic responses were examined in DIO and age-matched lean mice. In 16-wk-old lean mice, acute administration of rhFGF21 induced a dose-dependent increase in ERK phosphorylation in the liver and WAT with an ED50 value of 0.01 and 0.008 mg/kg, respectively (Fig. 7, A and B). A 3- to 4-fold maximum induction in ERK phosphorylation was observed in vivo; and were below plasma concentrations of rhFGF21 given at doses of 0.01 mg/kg or greater, ED50 doses for glucose, and body weight-lowering (Fig. 6).
FGF21 mRNA expression declined in the liver and WAT of lean and ob/ob mice after rhFGF21 treatment in the absence of increased expression of βKlotho and FGFR. A–F, mRNA expression levels of FGF21 (A), βKlotho (B), FGFR1c (C), FGFR2c (D), FGFR3c (E), and FGFR4 (F) in the liver (upper panels) and WAT (lower panels) of lean and ob/ob mice after rhFGF21 administration. Eight-week-old lean (solid line and closed circle) and ob/ob mice (dashed line and open circle) were ip administered with vehicle (0) or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice daily for 22 d. Total RNA were extracted and pooled RNA extracts from four animals per group were used for quantitative RT-PCR analysis. Data are normalized to cyclophilin control and expressed as relative mRNA levels compared with the value in vehicle-treated (0) lean mice, which was arbitrarily set to a value of 1.0. Results represent mean of duplicate RT-PCR analysis.

50% in the liver and WAT of DIO mice relative to those of lean mice (Fig. 7B). The attenuated induction in ERK phosphorylation was partly attributed to the elevated basal levels of p-ERK in the liver and WAT of DIO mice (Fig. 7A).

The in vivo metabolic effects of rhFGF21 were determined in 16-wk-old DIO and lean mice. After a single injection of 1 mg/kg rhFGF21 or vehicle, blood glucose, insulin, and amylin levels in rhFGF21-treated DIO mice were 31, 59, and 44% lower, respectively, than those in vehicle-treated DIO mice at the 2-h time point (Fig. 8, A–C). The effects were sustained for 4 h in DIO mice (Fig. 8, A–C). Glucose AUC over the 4-h period was reduced by 22% (P < 0.001), whereas the AUC of insulin and amylin were unchanged in rhFGF21-treated DIO mice compared with those in vehicle-treated DIO mice. In lean mice, acute treatment with rhFGF21 did not alter blood glucose levels (Fig. 8A). Although the plasma insulin and amylin levels were reduced at the 2-h time point (Fig. 8, B–C), the AUC of insulin and amylin over the 4-h period were not changed in rhFGF21-treated lean mice compared with those in vehicle-treated lean mice. The rhFGF21 had no effect on plasma glucagon levels in either lean or DIO mice (Fig. 8D).

Treatment of DIO mice with rhFGF21 dose dependently reduced blood levels of glucose (Fig. 8E) and insulin (Fig. 8F) and restored glucose tolerance (Fig. 8, I and J) to the levels seen in lean mice. The ED50 value was estimated to be 0.01 mg/kg for rhFGF21 to improve glycemia in DIO mice (Fig. 8F) and restored glucose tolerance (Fig. 8, I and J). In 16-wk-old lean mice, administration of rhFGF21 also dose dependently improved glucose tolerance (Fig. 8, I and J). Although a trend toward reduction in insulin levels was observed in 16-wk-old lean mice, the dose–response curve of insulin reduction was right shifted in lean mice relative to DIO mice (Fig. 8, F, I, and J). Plasma triglyceride levels in DIO mice were lower than those in lean mice receiving vehicle, and treatment with rhFGF21 reduced plasma triglyceride levels to a similar extent in lean and DIO mice (Fig. 8G). Plasma cholesterol levels were marginally reduced in DIO and lean mice (Fig. 8H). A substantially greater loss of body weight was observed in the DIO mice than those seen in lean mice.

FIG. 5. FGF21 mRNA expression declined in the liver and WAT of lean and ob/ob mice after rhFGF21 treatment in the absence of increased expression of βKlotho and FGFR. A–F, mRNA expression levels of FGF21 (A), βKlotho (B), FGFR1c (C), FGFR2c (D), FGFR3c (E), and FGFR4 (F) in the liver (upper panels) and WAT (lower panels) of lean and ob/ob mice after rhFGF21 administration. Eight-week-old lean (solid line and closed circle) and ob/ob mice (dashed line and open circle) were ip administered with vehicle (0) or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice daily for 22 d. Total RNA were extracted and pooled RNA extracts from four animals per group were used for quantitative RT-PCR analysis. Data are normalized to cyclophilin control and expressed as relative mRNA levels compared with the value in vehicle-treated (0) lean mice, which was arbitrarily set to a value of 1.0. Results represent mean of duplicate RT-PCR analysis.

FIG. 6. Plasma concentrations of rhFGF21 relative to endogenous FGF21 levels in lean and ob/ob mice. Ad libitum-fed, 8-wk-old lean (closed circle) and ob/ob mice (open circle) were ip administered with rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg. Blood samples were collected 15 min after injection, and plasma concentrations of rhFGF21 were analyzed. Data are means ± SEM (n = 3 per group). Mouse endogenous FGF21 concentrations were determined from the same cohort of lean and ob/ob mice receiving no rhFGF21 injection (n = 5 per group). Dashed lines indicate the mean concentration of mouse FGF21 in lean and ob/ob mice.
In this report we demonstrated that circulating FGF21 levels were elevated in two mouse models of obesity and insulin resistance, ob/ob and DIO mice. Additionally, the expression levels of FGF21 receptor components, including coreceptor βKlotho and signaling receptor FGFR1c and FGFR2c, were markedly down-regulated in the WAT of ob/ob and DIO mice. However, the FGF21 signaling cascade through ERK phosphorylation in the liver and WAT was retained in ob/ob mice and was partially attenuated in DIO mice. The in vivo metabolic effects of FGF21 were preserved in both ob/ob and DIO mice. The rhFGF21 exerted strong beneficial metabolic effects at supraphysiological concentrations, and elevated endogenous FGF21 levels likely represent a defense mechanism to combat obesity and insulin resistance.

A simultaneous occurrence of elevated circulating FGF21 levels and marked down-regulation of WAT βKlotho, FGFR1c, and FGFR2c expression was observed in both ob/ob and DIO mice. Relative to that seen in DIO mice, ob/ob mice showed severely increased plasma concentrations of FGF21 and reduced βKlotho protein content. If elevated circulating FGF21 levels are indicative of FGF21 resistance, ob/ob mice would be expected to be more FGF21 resistant than DIO mice. This hypothesis was not supported by our results. The FGF21 signaling cascade through ERK phosphorylation was largely retained in the liver and WAT of ob/ob mice despite reduced WAT βKlotho and FGFR levels. The dose-response curves were not rightward shifted, and the maximum inductions in ERK phosphorylation were also comparable between ob/ob and lean mice. The kinetics of ERK phosphorylation in the WAT was also similar in lean and ob/ob mice. In DIO mice, the dose-response curves on ERK phosphorylation were not shifted to the right; however, the maximum inductions in ERK phosphorylation in the liver and WAT were attenuated by approximately 50% at the maximum rhFGF21 dose levels compared with those in lean mice. The response in ERK phosphorylation did not appear to be correlated with the tissue expression levels of βKlotho and FGFR in either ob/ob or DIO mice. In addition, the signaling defect observed in DIO mice was likely animal model specific and occurred at the postreceptor level because it was observed in the liver of DIO mice in the presence of normal expression levels of βKlotho and FGFR.

The marked reduction in WAT βKlotho and FGFR did not cause a diminished ERK phosphorylation at submaximal doses of rhFGF21 in either ob/ob or DIO mice. One possible explanation may relate to the unique mode of FGF21 activation of the receptor complex consisting of βKlotho and FGFR (2, 23). βKlotho is a coreceptor that binds to FGF21 and facilitates FGF21 activation of FGFR (3–5). However, a direct interaction between FGF21 and FGFR has not been demonstrated, even in the presence of βKlotho (4), suggesting that FGF21 activation of FGFR is highly cooperative and may be transient. It is therefore possible that 10% remaining βKlotho in diseased animals were sufficient to facilitate the cooperation between FGF21 and FGFR, and a full occupancy of FGFR may be not necessary to trigger a full response. Alternatively, our data appeared to align with a report indicating that FGF21 had βKlotho-independent activity in vivo (24). In βKlotho knockout mice, FGF21 was found fully capable of inducing early growth response protein 1 expression in the

**Discussion**

In this report we demonstrated that circulating FGF21 levels were elevated in two mouse models of obesity and insulin resistance, ob/ob and DIO mice. Additionally, the expression levels of FGF21 receptor components, including coreceptor βKlotho and signaling receptor FGFR1c and FGFR2c, were markedly down-regulated in the WAT of ob/ob and DIO mice. However, the FGF21 signaling cascade through ERK phosphorylation in the liver and WAT was retained in ob/ob mice and was partially attenuated in DIO mice. The in vivo metabolic effects of FGF21 were preserved in both ob/ob and DIO mice. The rhFGF21 exerted strong beneficial metabolic effects at supraphysiological concentrations, and elevated endogenous FGF21 levels likely represent a defense mechanism to combat obesity and insulin resistance.

A simultaneous occurrence of elevated circulating FGF21 levels and marked down-regulation of WAT βKlotho, FGFR1c, and FGFR2c expression was observed in both ob/ob and DIO mice. Relative to that seen in DIO mice, ob/ob mice showed severely increased
WAT. A compensatory increase in circulating FGF21 levels was also not observed in βKlotho knockout mice. Taken together, the in vivo role of βKlotho in the FGF21 pathway requires further elucidation.

Endocrine hormone resistance is common. Typical examples include insulin and leptin resistance. In general, the molecular defects at the receptor or postreceptor levels correlate with attenuated whole-body responses (25). In the case of insulin resistance, diabetic humans and animals are less responsive to injected insulin, and higher doses of insulin are required to achieve adequate glucose control (26). In the case of leptin resistance, little response could be achieved, even when leptin was given at high doses in obese mice (27). Here blood glucose levels in ob/ob and DIO mice were reduced to normal levels within 2 h of rhFGF21 injection. The magnitude of acute reduction in glucose, insulin, and amylin levels was highest in ob/ob mice, intermediate in DIO mice, and lowest in lean mice. The ED₅₀ value of rhFGF21 was estimated to be 0.01 mg/kg for reducing blood glucose levels and improving glucose tolerance in ob/ob and DIO mice, whereas treatment of lean mice with rhFGF21 up to 1 mg/kg did not show a consistent glucose-lowering effect. Plasma insulin levels were reduced in diseased mice at a dose as low as 0.001 mg/kg, whereas insulin levels trended down in lean mice receiving rhFGF21 at doses of 0.1 mg/kg or greater. Body weight reduction was also substantially greater in DIO mice than in lean mice. Among all the parameters tested, only the response in triglyceride reduction appeared more sensitive in lean mice than in diseased mice. Plasma and hepatic triglycerides were reduced in lean mice receiving rhFGF21 at a dose as low as 0.001 mg/kg, whereas a reduction in plasma and hepatic triglyceride levels in diseased mice required rhFGF21 at a dose of 0.1 mg/kg or greater. Although the in vivo response may reflect a constellation of FGF21 action at multiple target organs, and its interactions with different physiological or pathophysiological systems, our data suggest that FGF21 resistance is not apparent in either ob/ob or DIO mice at the whole-organism level.

Although circulating FGF21 levels were elevated in ob/ob mice relative to those in lean mice, the FGF21 levels in ob/ob mice were similar to plasma concentrations of rhFGF21 administered at 0.001 mg/kg, a starting effective

FIG. 8. Acute and subchronic metabolic effects of rhFGF21 in lean and DIO mice. A–D, Ad libitum-fed, 16-wk-old mice were ip injected with vehicle (closed circles) or 1 mg/kg rhFGF21 (open circles). Blood glucose (A) and plasma levels of insulin (B), amylin (C), and glucagon (D) were measured before injection (0 h) and 2 and 4 h after injection. Data are means ± SEM (n = 12 per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with vehicle-treated group at each time point. E–L, Dose-response effects of rhFGF21 on metabolic parameters in lean and DIO mice. Sixteen-week-old lean and DIO mice were ip administered with vehicle (0) or rhFGF21 at doses of 0.001, 0.01, 0.1, or 1 mg/kg twice daily for 10 d. Blood samples were collected from ad libitum-fed mice on study d 7 at 1 h after the morning injection. Blood glucose (E) and plasma levels of insulin (F), triglyceride (G), and cholesterol (H) were measured. I and J, A GTT was conducted on study d 10 at 1 h after the morning injection (I) and glucose AUC are shown in J. K, Body weight change in lean and DIO mice over a 10-d period. L, Percent change in body weight on d 10. All data are means ± SEM (n = 8 per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with vehicle-treated lean or DIO mice.
dose observed in vivo, and were below plasma concentrations of rhFGF21 administered at 0.01 mg/kg or greater, ED_{50} values for improving glycemia and reducing body weight. Assuming that rhFGF21 has biological activity equivalent to mouse FGF21, these data suggest that the diseased mice are still in a state of relative FGF21 deficiency. Supraphysiological levels of FGF21 or pharmacological intervention is likely required to alleviate diabetes and obesity.

Our results contradict those of a report showing that DIO mice were in a state of FGF21 resistance (28). The in vivo discrepancy may be related to the selection of different functional readouts for FGF21. We did not use parameters related to lipolysis because the role of FGF21 in lipolysis is still controversial (13, 29, 30). Our assessment was focused on well-validated FGF21 actions on glucose, triglyceride, and body weight reduction. Additionally, we purposely chose a maximum dose of rh-FGF21 to assess acute response of FGF21 in vivo. Studies with a wide-range dose response were also conducted to thoroughly evaluate FGF21 sensitivity and responsiveness. The molecular mechanism underlying the elevated FGF21 levels in the obese and insulin-resistant state requires further elucidation. The elevated circulating FGF21 levels paralleled the increased FGF21 mRNA expression in the liver and also correlated with a small increased FGF21 mRNA expression in the WAT. The increase in FGF21 mRNA expression was liver and WAT specific because pancreatic FGF21 expression was dramatically reduced in ob/ob and DIO mice. The alteration in FGF21 expression was likely acquired when obesity and insulin resistance developed, because it was observed in DIO mice, which were normal mice fed a high-fat diet. The degree of alteration in FGF21 expression was more pronounced in ob/ob mice than in DIO mice, which was in a general agreement with the severity of obesity and diabetes associated with these two models. Collectively our data suggest that elevated circulating FGF21 levels were derived from increased FGF21 mRNA expression in the liver with a small contribution from the WAT as obesity and insulin resistance developed. In the obese, insulin-resistant state, fat accumulation increases in the metabolic tissues. The increased fat availability may activate PPAR\_y in the WAT, resulting in increased FGF21 transcription followed by protein synthesis and secretion. This hypothesis is further supported by the observation that FGF21 mRNA expression declined in the liver and WAT as plasma and hepatic triglyceride levels decreased or as adiposity and insulin resistance improved after rh-FGF21 treatment. The decline in FGF21 mRNA expression occurred in the absence of increased expression of either βKlotho or FGFR.

Pharmacological administration of rhFGF21 at levels 1000-fold higher than the endogenous levels did not reduce mRNA expression levels of βKlotho, FGFR1c, FGFR3c, or FGFR4, suggesting that the down-regulation of WAT βKlotho and FGFR1c may not be FGF21 dependent. FGFR2c expression levels were dose dependently reduced in the WAT of lean mice in response to rhFGF21 treatment suggesting that FGFR2c may be the preferred receptor for FGF21 in vivo.

In summary, we have provided evidence that FGF21 resistance was not evident at the whole-organism level in animal models of genetic or acquired obesity and insulin resistance. Further investigation on the molecular mechanisms that govern FGF21, βKlotho, and FGFR expression in the obese and insulin-resistant states is warranted.

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