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Adiponectin Mediates the Metabolic Effects of FGF21 on Glucose Homeostasis and Insulin Sensitivity in Mice

Zhuofeng Lin,[1] Haishan Tian,[1] Karen S.L. Lam,[2] Shaoqiang Lin,[1] Ruby C.L. Hoo,[2] Morichika Konishi,[4] Nobuyuki Itoh,[4] Yu Wang,[3] Stefan R. Bornstein,[5] Aimin Xu,[1,2,3,*] and Xiaokun Li[1,*]
[1]Institute of Metabolic Medicine, School of Pharmacology, Wenzhou Medical College, Wenzhou 325035, China
[2]Department of Medicine
[3]Department of Pharmacology and Pharmacy
The University of Hong Kong, Hong Kong, China
[4]Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Sakyo, Kyoto 606-8501, Japan
[5]Department of Medicine, University of Dresden, 01307 Dresden, Germany
*Correspondence: amxu@hku.hk (A.X.), xiaokunli@163.net (X.L.)
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SUMMARY

Fibroblast growth factor 21 (FGF21) is a metabolic hormone with pleiotropic effects on regulating glucose and lipid homeostasis and insulin sensitivity. However, the mechanisms underlying the metabolic actions of FGF21 remain unknown. Here we show that the insulin-sensitizing adipokine adiponectin is a downstream effector of FGF21. Treatments with FGF21 enhanced both expression and secretion of adiponectin in adipocytes, thereby increasing serum levels of adiponectin in mice. Adiponectin knockout mice were refractory to several therapeutic benefits of FGF21, including alleviation of obesity-associated hyperglycemia, hypertriglyceridemia, insulin resistance, and hepatic steatosis. Furthermore, the effects of FGF21 on attenuation of obesity-induced impairment in insulin signaling in liver and skeletal muscle were abrogated in adiponectin knockout mice, whereas FGF21-mediated activation of ERK1/ERK2 in adipose tissues remained unaffected. Therefore, adiponectin couples FGF21 actions in local adipocytes to liver and skeletal muscle, thereby mediating the systemic effects of FGF21 on energy metabolism and insulin sensitivity.

INTRODUCTION

Fibroblast growth factor 21 (FGF21) is a member of the endocrine FGF subfamily with multiple metabolic functions (Potthoff et al., 2012; Kharitonenkov et al., 2005). Unlike the prototypical members of the FGF family, FGF21 does not have mitogenic activities but acts as an important regulator of energy homeostasis, glucose and lipid metabolism, and insulin sensitivity. Physiologically, FGF21 plays a key role in mediating the metabolic responses to fasting or starvation, including fatty acid oxidation and ketogenesis (Badman et al., 2007; Potthoff et al., 2009; Inagaki et al., 2007). Pharmacologically, recombinant FGF21 therapies have been shown to counteract obesity and its related metabolic disorders in both rodents and nonhuman primates, including reduction of adiposity and alleviation of hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, and fatty liver disease (Xu et al., 2009b; Coskun et al., 2008; Kharitonenkov et al., 2007). Furthermore, FGF21 is the downstream target of both peroxisome proliferator-activated receptor-alpha (PPARα) and PPARγ, and a growing body of evidence suggest that the glucose-lowering and insulin-sensitizing effects of the PPARγ agonists (thiazolidinediones, TZDs) and the therapeutic benefits of the PPARα agonists (fenofibrates) on lipid profiles are mediated in part by induction of FGF21 (Dutchak et al., 2012; Li et al., 2012).

While the liver is the predominant site for FGF21 production, adipocytes are a main target of FGF21 actions (Ding et al., 2012; Véniant et al., 2012; Adams et al., 2013; Chen et al., 2011b). In white adipocytes, FGF21 stimulates glucose uptake in an insulin-independent manner (Kharitonenkov et al., 2005), modulates lipolysis (Chen et al., 2011b), enhances mitochondrial oxidative capacity (Chau et al., 2010), and potentiates PPARγ activity (Dutchak et al., 2012). There is also good evidence that FGF21 is involved in the thermogenic functions of brown adipocytes (Hondares et al., 2011; Potthoff et al., 2012). Furthermore, FGF21 is expressed and secreted in both white and brown adipocytes (Hondares et al., 2011; Zhang et al., 2008), and the autocrine actions of FGF21 in adipocytes play an obligatory role in mediating the metabolic benefits of PPARγ on glucose homeostasis and peripheral insulin sensitivity, by forming a feed-forward loop with this nuclear receptor (Dutchak et al., 2012). Notably, lipodystrophic mice with reduced adipose tissue are refractory to both acute and chronic effects of systemic FGF21 administration in decreasing blood glucose and in increasing insulin sensitivity, whereas the FGF21 responsiveness was completely restored after transplantation of white adipose tissue into lipodystrophic mice, thus confirming that adipose tissue is a predominant site conferring the antidiabetic activities of FGF21 (Véniant et al., 2012). However, it is currently unclear how FGF21 controls systemic metabolic homeostasis via its actions in adipocytes.

Adiponectin, an adipokine predominantly secreted from adipocytes, bears many functional similarities with FGF21 in

regulating glucose and lipid metabolism and insulin sensitivity. In different animal models of obesity and diabetes, adiponectin decreases hyperglycemia, alleviates glucose intolerance and insulin resistance, improves lipid profiles, and ameliorates fatty liver disorders (Yamauchi et al., 2001; Xu et al., 2003; Berg et al., 2001). Adiponectin controls systemic glucose and lipid homeostasis via its endocrine actions on several major metabolic organs, including liver and skeletal muscle (Kadowaki et al., 2006). Furthermore, adiponectin is also a downstream effector of PPARγ and is an essential mediator for many therapeutic benefits of the PPARγ agonists TZDs, including insulin sensitization and vascular protection (Nawrocki et al., 2006; Chang et al., 2010; Kubota et al., 2006).

In this study, we investigated the reciprocal relationship between FGF21 and adiponectin in both primary adipocytes and mice and tested our hypothesis that adiponectin may mediate the pleiotropic metabolic benefits of FGF21 by comparing both acute and chronic effects of recombinant mouse FGF21 (rmFGF21) in adiponectin knockout (ADNKO) mice and wild-type (WT) controls with dietary or genetic obesity.

RESULTS

FGF21 Increases Both Adiponectin Expression and Secretion in Adipocytes

FGF21 and adiponectin exhibit similar beneficial effects on metabolism and insulin sensitivity. To elucidate the relationship between these two metabolic regulators, we first investigated the effects of FGF21 on adiponectin expression and secretion. Treatment of mouse adipocytes with rmFGF21 caused a significant elevation of adiponectin secretion in a time-dependent manner (Figure 1A). At 1, 6, 12, and 24 hr after treatment, total adiponectin concentrations in the conditioned medium were 1.6- ± 0.3-, 2.1- ± 0.3-, 2.4- ± 0.4-, and 2.6- ± 0.5-fold higher than the vehicle control group, respectively. On the other hand, a progressive increase in adiponectin messenger RNA (mRNA) expression occurred from 6 hr after FGF21 treatment (Figure 1B). Pulse-chase experiment with ^{35}S methionine demonstrated that FGF21 markedly enhanced the secretion rate of newly synthesized adiponectin from adipocytes (Figure 1C), suggesting that FGF21 increases both the gene transcription and secretion of adiponectin.

As FGF21 has been shown to activate PPARγ in adipocytes (Dutchak et al., 2012), we next investigated the role of this transcription factor in FGF21-induced adiponectin production. Pretreatment of mouse adipocytes with the PPARγ antagonist GW9662 partially abrogated FGF21-induced secretion of adiponectin (Figure 2A) and also significantly decreased FGF21-induced adiponectin mRNA expression (Figure 2B). Likewise, the magnitude of FGF21-stimulated adiponectin secretion and gene expression in fat explants isolated from PPARγ$^{+/-}$ mice was much lower than those from wild-type littermates (Figures 2C and 2D). Furthermore, the protein expression of Ero1-Lα and DsbA-L, both of which are molecular chaperones involved in adiponectin oligomerization and secretion (Wang et al., 2007; Liu et al., 2008), was significantly elevated by FGF21 treatments, whereas such an effect of FGF21 was attenuated by GW9662 or PPARγ haplodeficiency (Figures 2E and 2F).

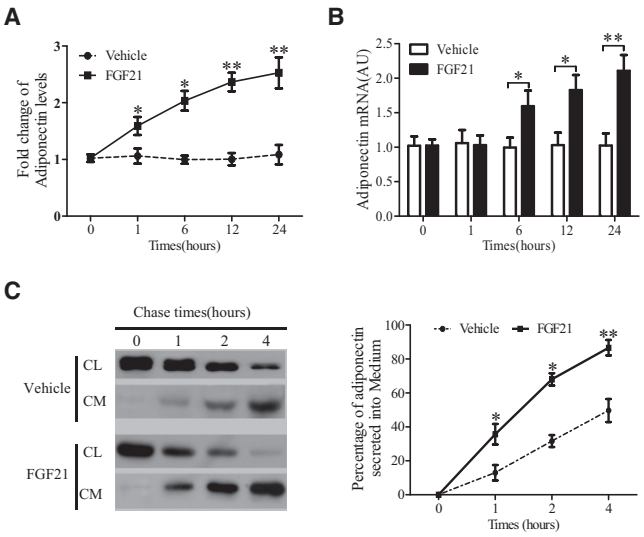

Figure 1. The Effects of FGF21 on Expression and Secretion of Adiponectin in Mouse Adipocytes

Stromal vascular cells isolated from epididymal fat pads were differentiated into mature adipocytes, which were then treated with 1 μg/ml recombinant mouse FGF21 (rmFGF21) or vehicle control for various periods.

(A) Total adiponectin concentrations in serum-free medium, expressed as the fold over vehicle control in each time point.

(B) The relative mRNA abundance of the FGF21 gene as determined by real-time PCR.

(C) Representative autoradiograph of ^{35}S-labeled adiponectin at various stages during a pulse-chase study. Cell lysates (CL) and conditioned medium (CM) at different time intervals after the chase were collected and subjected to immunoprecipitation using rabbit anti-mouse adiponectin IgG. The graph in the right panel represents the percentage of adiponectin released into the culture media.

Data are presented as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ versus vehicle control. n = 5 in each group. See also Table S2.

Adiponectin Production in Adipocytes Is Induced by Autocrine Actions of FGF21

In addition to hepatocytes, FGF21 is also expressed in both mouse and human adipocytes (Zhang et al., 2008). Adipocyte-derived FGF21 has been implicated in the insulin-sensitizing activity of the PPARγ agonists (Dutchak et al., 2012). We next investigated whether endogenous FGF21 in adipocytes modulates adiponectin production in an autocrine manner. FGF21-null adipocytes exhibited a modest decrease in both adiponectin mRNA expression (Figure 3A) and its protein concentration in the conditioned medium compared to WT controls (Figure 3B). Furthermore, the amplitude of rosiglitazone-induced adiponectin expression and secretion was markedly attenuated in FGF21-null adipocytes (Figures 3A and 3B). Likewise, the stimulatory effects of rosiglitazone on adiponectin production in the explants of human visceral fat pads were also diminished by coincubation with a neutralizing antibody against FGF21 (Figure 3C).

FGF21 Acts as an Upstream Regulator of Adiponectin Production in Mice

Consistent with our in vitro findings above, the serum level of total adiponectin in FGF21 KO mice was significantly lower

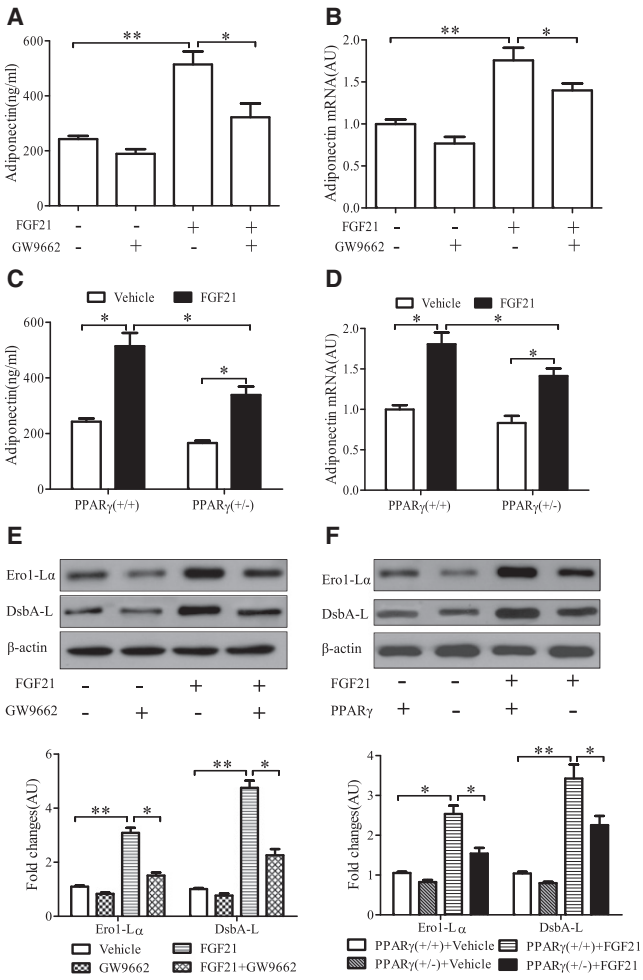

Figure 2. The Role of PPARγ in FGF21-Induced Expression and Secretion of Adiponectin

Mouse primary adipocytes differentiated from stromal vascular cells were pretreated with GW9662 (10.0 μM) for 1 hr, followed by incubation with FGF21 (1 μg/ml) for another 6 hr. Adiponectin concentration in the conditioned medium (A) and relative adiponectin mRNA abundance (B) were analyzed as in Figure 1. (C) and (D) show the comparison of adiponectin concentration in the conditioned medium and adiponectin mRNA abundance between PPARγ$^{+/-}$ and PPARγ$^{+/+}$ mouse adipocytes treated with or without FGF21 (1 μg/ml) for 6 hr. (E) and (F) show the representative immunoblots for the protein expression levels of Ero1-Lα and DsbA-L in mouse adipocytes treated as in (A), or PPARγ$^{+/-}$ and PPARγ$^{+/+}$ mouse adipocytes as in (C). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01. n = 5–6. See also Table S2.

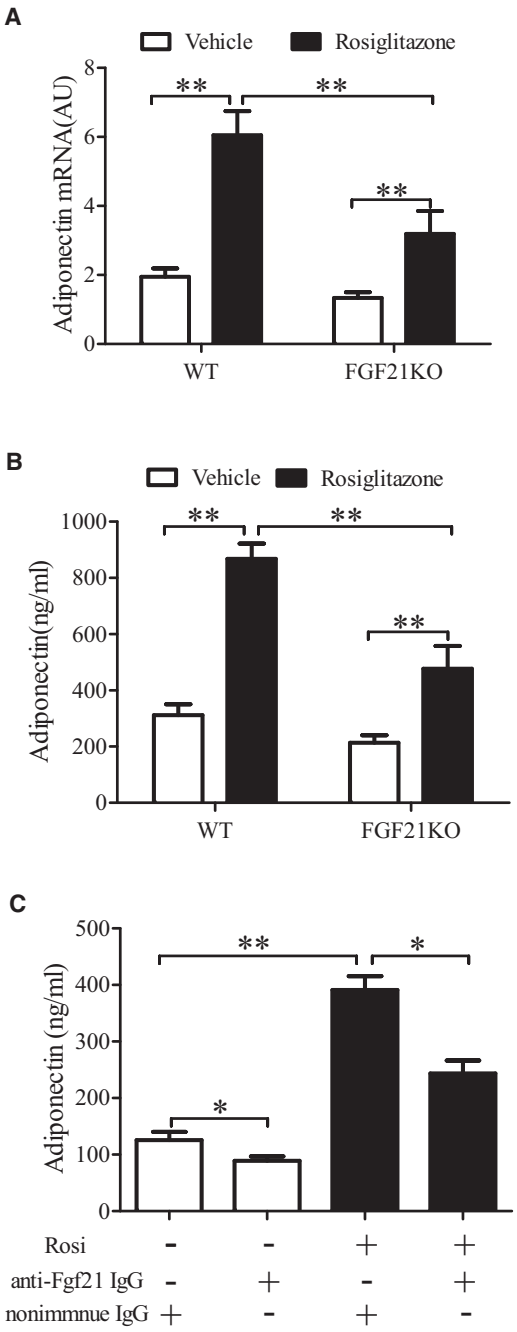

Figure 3. The Roles of Endogenous FGF21 in the PPARγ Agonist-Induced Adiponectin Production in Adipocytes

(A and B) Mature adipocytes derived from stromal vascular cells isolated from FGF21 KO and WT mice were treated with rosiglitazone (1.0 μM) for 6 hr in a serum-free DMEM medium. The relative mRNA abundances of the adiponectin gene (A) and adiponectin protein concentration (B) in the conditioned medium were analyzed in Figure 1.

(C) Adiponectin protein concentration in the conditioned medium of human adipose tissue explants treated with rosiglitazone (1.0 μM) in the presence of rabbit anti human FGF21 IgG (5 μg/ml) or nonimmune IgG as a control for 6 hr.

Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01. n = 5 in each group.

than that in age-matched wild-type mice on standard chow. Notably, high-fat diet (HFD) feeding for 4 weeks increased serum levels of both FGF21 and adiponectin in WT mice, whereas such a HFD-induced elevation in adiponectin was largely abrogated in FGF21 KO mice (Figure 4A). At 35 weeks after feeding with HFD, plasma levels of adiponectin in wild-type mice was markedly decreased as compared to mice fed standard chow (Figure 4A). Furthermore, the magnitude of decrease in circulating adiponectin in HFD-fed FGF21 knockout mice was much greater than that in HFD-fed wild-type mice, demonstrating that the lack of FGF21 leads to a further reduction of adiponectin. Acute injection of recombinant FGF21 led to a significant elevation of circulating

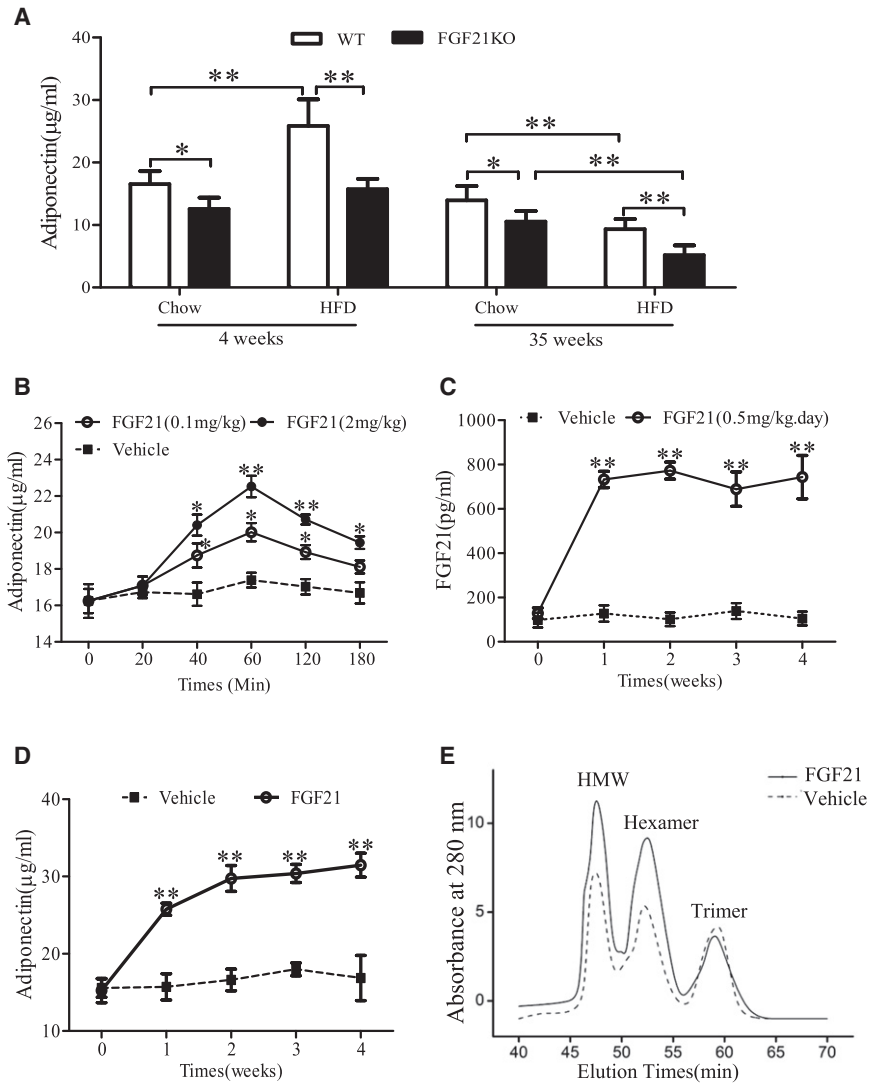

Figure 4. FGF21 Induces Adiponectin Production in Mice

(A) Serum levels of adiponectin in FGF21 KO and WT mice on chow and HFD for 4 weeks and 35 weeks.

(B) The acute effect of single rmFGF21 injection (2 mg/kg and 0.1 mg/kg) on serum levels of adiponectin in 10-week-old C57/BL6 mice.

(C) The circulating levels of FGF21 at various time points after mice receiving chronic delivery of rmFGF21 with subcutaneously implanted osmotic pumps.

(D) Effects of chronic administration with rmFGF21 on serum levels of adiponectin.

(E) The distribution of the three oligomeric complexes of adiponectin as determined by gel filtration analysis. Serum samples were collected from the mice after treatment with rmFGF21 for 4 weeks.

Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus vehicle control group in each time point. n = 5 in each group.

adiponectin (Figure 4B). Likewise, chronic administration of recombinant FGF21 with an osmotic pump (0.5 mg/kg per day) resulted in an approximately 5-fold elevation of circulating FGF21 over the endogenous level (Figure 4C), and such a change was accompanied by an ~2-fold increase in plasma levels of adiponectin (Figure 4D). Further gel filtration analysis demonstrated that both high-molecular-weight and hexameric adiponectin were significantly elevated after FGF21 treatment, although the magnitude of increase in the high-molecular-weight adiponectin was more obviously than that of the hexameric oligomers (Figure 4E).

Adiponectin Mediates the Therapeutic Benefits of FGF21 on Hyperglycemia and Insulin Resistance

Adipocytes play an obligatory role in conferring the pleiotropic effects of FGF21 on glucose and lipid metabolism (Véniant et al., 2012). In light of the strikingly overlapping functions between FGF21 and adiponectin, we next investigated whether adiponectin acts as a downstream effector mediating the acute and/or chronic metabolic effects of FGF21 in mice. Consistent with previous reports (Berglund et al., 2009), single injection of recombinant FGF21 acutely decreased blood levels of glucose, insulin, and triglyceride in HFD-induced obese mice (Figures 5A–5C), as well as in db/db diabetic mice (Figure S1 available online). In ADNKO mice with dietary and genetic obesity, acute treatment with FGF21 was also able to decrease glucose, insulin, and triglycerides levels. However, the magnitude of FGF21-mediated decreases in ADNKO mice was significantly lower than those in wild-type controls, suggesting that these acute metabolic effects of FGF21 are mediated in part by adiponectin. On the other hand, FGF21-induced phosphorylation of ERK1/ERK2 in adipose tissue was comparable between ADNKO mice and WT controls (Figure 5D). The expression levels of the four major types of FGF receptors (FGFR1–FGFR4) in adipose tissues remained unchanged, whereas there was a modest, but significant, elevation in β-klotho expression in ADNKO mice as compared to wild-type controls (Figure S2).

We next compared the chronic effects of FGF21 in ADNKO mice and WT littermates with diet-induced obesity using an osmotic pump-based delivery system. Chronic treatment with recombinant FGF21 for a period of 4 weeks caused a comparable level of decrease in diet-induced body weight and fat mass gains between WT and ADNKO mice (Figures 6A and 6B). A glucose tolerance test demonstrated that chronic administration of FGF21 into WT mice ameliorated HFD-induced impairment in glucose clearance (Figure 6C). HFD-induced insulin resistance, as determined by an insulin tolerance test, was also dramatically attenuated by chronic FGF21 treatment (Figure 6D). At the molecular level, such improvements in glucose clearance and insulin sensitivity in FGF21-treated obese mice were accompanied by a significant elevation in insulin-stimulated phosphorylation of

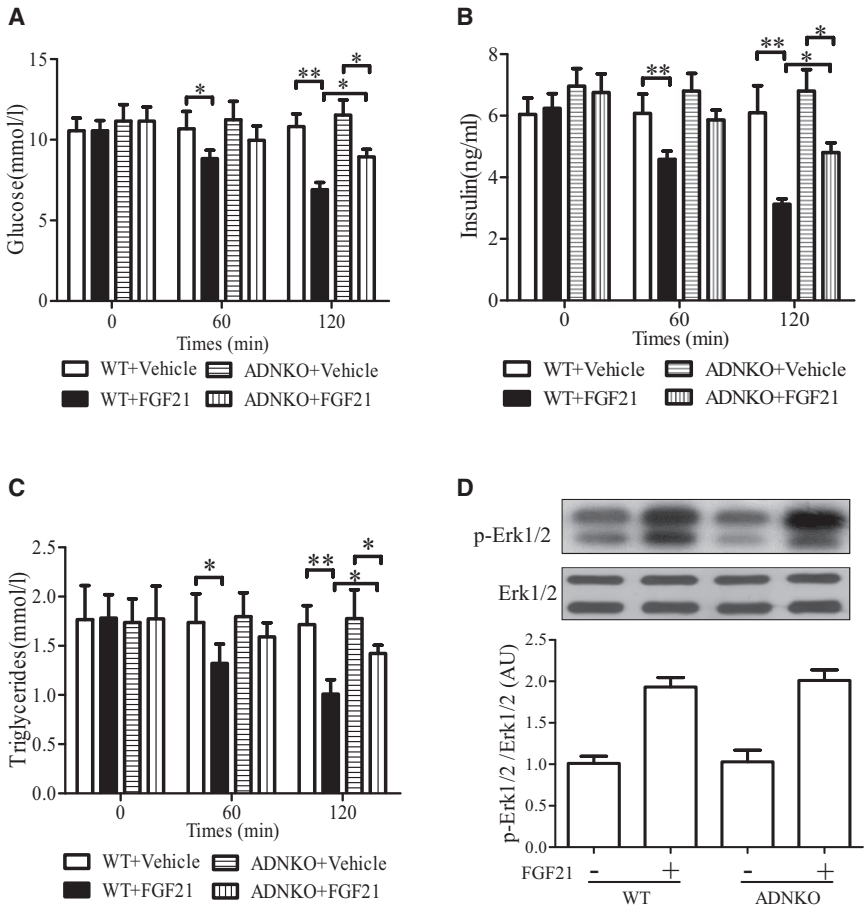

Figure 5. The Acute Metabolic Effects of rmFGF21 in Mice with or without Adiponectin

(A–C) Blood glucose levels (A), serum insulin concentrations of insulin (B), and triglycerides (C) were measured in HFD-fed ADNKO and WT mice at various time points (0, 60, and 120 min) after single injection with rmFGF21 (2mg/Kg).

(D) Representative western blots showing the effects of acute FGF21 administration on total and phosphorylated ERK1/ERK2 in epididymal fat. The fat pads were collected at 1 hr after injection with rmFGF21.

Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 6 in each group. See also Figures S1 and S2.

Akt and its downstream target, glycogen synthase kinase 3β (GSK3β), as compared with those in vehicle-treated obese mice (Figure 6E). By contrast, all these metabolic benefits of chronic FGF21 administration, including alleviation of HFD-induced glucose intolerance, insulin resistance, and impaired insulin-evoked Akt activation in the liver, were largely abrogated in ADNKO mice (Figure 6F).

Adiponectin Contributes to the Metabolic Benefits of FGF21 in Both Liver and Skeletal Muscle

Since both therapeutic administration and transgenic expression of FGF21 has been shown to improve serum lipid profiles and alleviate fatty liver in mice and nonhuman primates (Xu et al., 2009b; Kharitonenkov et al., 2007), we next investigated whether such chronic effects of FGF21 are dependent on adiponectin. In both ADNKO mice and WT controls on HFD, chronic FGF21 administration caused a similar degree of reduction in serum levels of total cholesterol (Figure 7A). Serum levels of total triglycerides in C57 WT mice were decreased by 57% after chronic treatment with FGF21, whereas this FGF21-mediated reduction was largely abrogated in ADNKO mice (Figure 7B).

Hematoxylin and eosin (H&E) analysis of liver sections demonstrated that HFD-induced hepatic steatosis in C57 mice was substantially attenuated by systemic administration of FGF21 for 4 weeks (Figure 7C), and this change was accompanied by a marked reduction in serum levels of the liver injury marker alanine aminotransferase (ALT) (Figure 7D) and decreased expression of the proinflammatory cytokines TNF-α and MCP-1 (Figure 7E). By contrast, the beneficial effects of chronic FGF21 administration on HFD-induced hepatic steatosis, ALT elevation, and expression of proinflammatory cytokines were largely diminished in adiponectin-deficient mice. In WT mice, systemic administration of FGF21 significantly suppressed the expression of several genes involved in lipogenesis (ACC1, ACC2, CYP8B1, SCD1, and fatty acid synthase; Table S1), whereas such effects of FGF21 were abrogated in ADNKO mice. By contrast, FGF21 inhibited the expression of 3-hydroxy-3-methylglutaryl CoA reductase (a rate-limiting enzyme of cholesterol synthesis) to a comparable level in ADNKO mice and WT controls.

Consistent with the observations in the liver, chronic FGF21 administration significantly decreased the intramuscular triglyceride accumulation and increased insulin-evoked Akt activation in both soleus and gastrocnemius muscles and of HFD-induced obese mice, whereas these muscular effects of FGF21 were abrogated in ADNKO mice (Figure S3).

Adiponectin Confers the Effects of FGF21 on Hepatic Fatty Acid Oxidation and Lipid Clearance

Chronic treatment with FGF21 had no obvious effect on food intake in either ADNKO mice or WT controls (Figure S4A). In WT mice chronically administered with FGF21, energy expenditure (VO_2) was increased, whereas the respiratory exchange ratio (RER) was decreased, as compared to vehicle-treated group (Figures S4B–S4D). In ADNKO mice, FGF21-induced elevation of energy expenditure was comparable to WT mice, whereas the magnitude of FGF21-mediated RER reduction was significantly smaller than that in WT mice, suggesting that FGF21-induced lipid utilization is partly dependent on adiponectin. Chronic FGF21 administration decreased serum levels of free fatty acids (FFAs) and increased serum concentrations of β-hydroxybutyrate (Figures S5A and S5B), whereas the

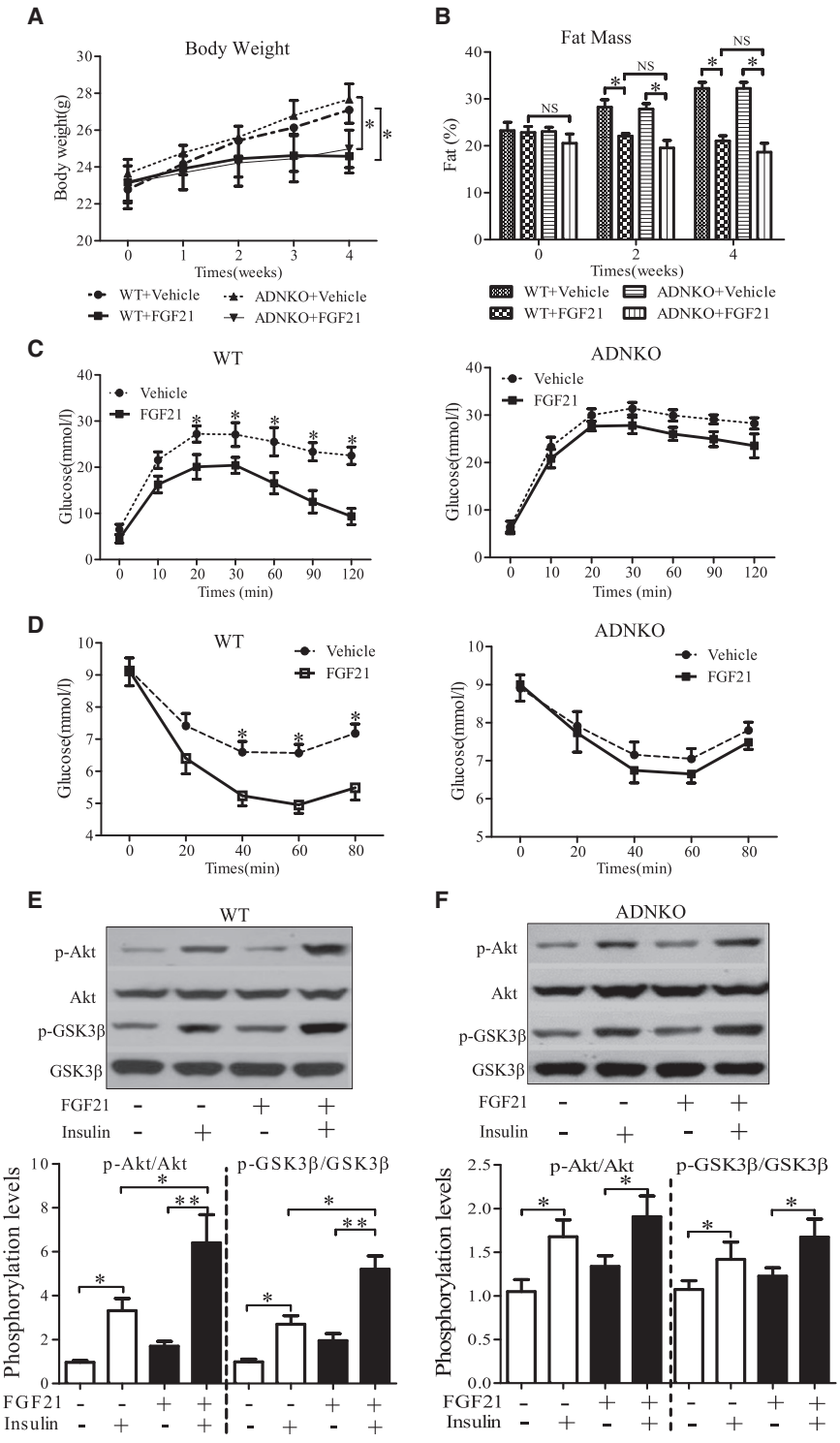

Figure 6. Effects of Chronic rmFGF21 Administration on Body Weight, Fat Mass, Glucose Metabolism, and Insulin Sensitivity in Mice with or without Adiponectin

Four-week-old ADNKO and WT mice were fed with HFD for 4 weeks, followed by systemic administration with rmFGF21 or saline (as a vehicle control) with osmotic pumps (0.5 mg/kg per day) for another 4 weeks.

(A) Body weight measured weekly after treatment

(B) Fat mass measured biweekly.

(C) GTT performed at 2 weeks after rmFGF21 treatment.

(D) ITT performed at 4 weeks after rmFGF21 treatment.

(E and F) Representative immunoblots showing phospho (Thr308) and total Akt, phospho (Ser9) and GSK3β in the liver of WT and ADNKO mice, respectively. The tissues were collected at 10 min after single injection of insulin (0.5 U/kg) in mice on HFD treated with rmFGF21 or vehicle for 2 weeks. The bar chart under each blot is the densitometric analysis of phosphorylation levels.

Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 5 in each group. NS, not significant. See also Figures S3–S5 and Table S1.

amplitude of FGF21-mediated reduction in FFAs and elevation in β-hydroxybutyrate in ADNKO mice was markedly attenuated. The rate of [1-^{14}C]palmitate oxidation in the liver homogenate freshly isolated from FGF21-treated WT mice was significantly higher than vehicle-treated controls, but this FGF21-induced elevation was compromised in ADNKO mice (Figure S5C). Similarly, chronic treatment of primary hepatocytes with recombinant adiponectin, but not FGF21, increased [1-^{14}C]palmitate oxidation (Figure S5D), suggesting that FGF21-induced hepatic fatty acid oxidation is not due to its direct effect on hepatocytes. In response to oral challenge with peanut oil, WT mice receiving chronic treatment of FGF21 exhibited significantly higher rates of lipid clearance as compared to the vehicle-treated mice. On the other hand, ADNKO mice displayed severely impaired lipid clearance under both basal and FGF21-treated conditions when comparing to the WT littermates (Figure S5E).

DISCUSSION

Although both pharmacological and physiological studies in rodents and nonhuman primates have demonstrated the profound effects of FGF21 in controlling glucose and lipid homeostasis, the underlying mechanisms remain poorly characterized. The tissue specificity of FGF21 actions is determined by the expression of FGFR1 and the single-pass membrane protein β-klotho, which have been shown to act as a receptor and coreceptor of FGF21, respectively (Kharitonenkov et al., 2008). Adipocytes, in which both FGFR1 and β-klotho are highly expressed, are a predominant site of FGF21 action. Data from both lipodystrophic mice (Véniant et al., 2012) and adipose-specific FGFR1 (Adams

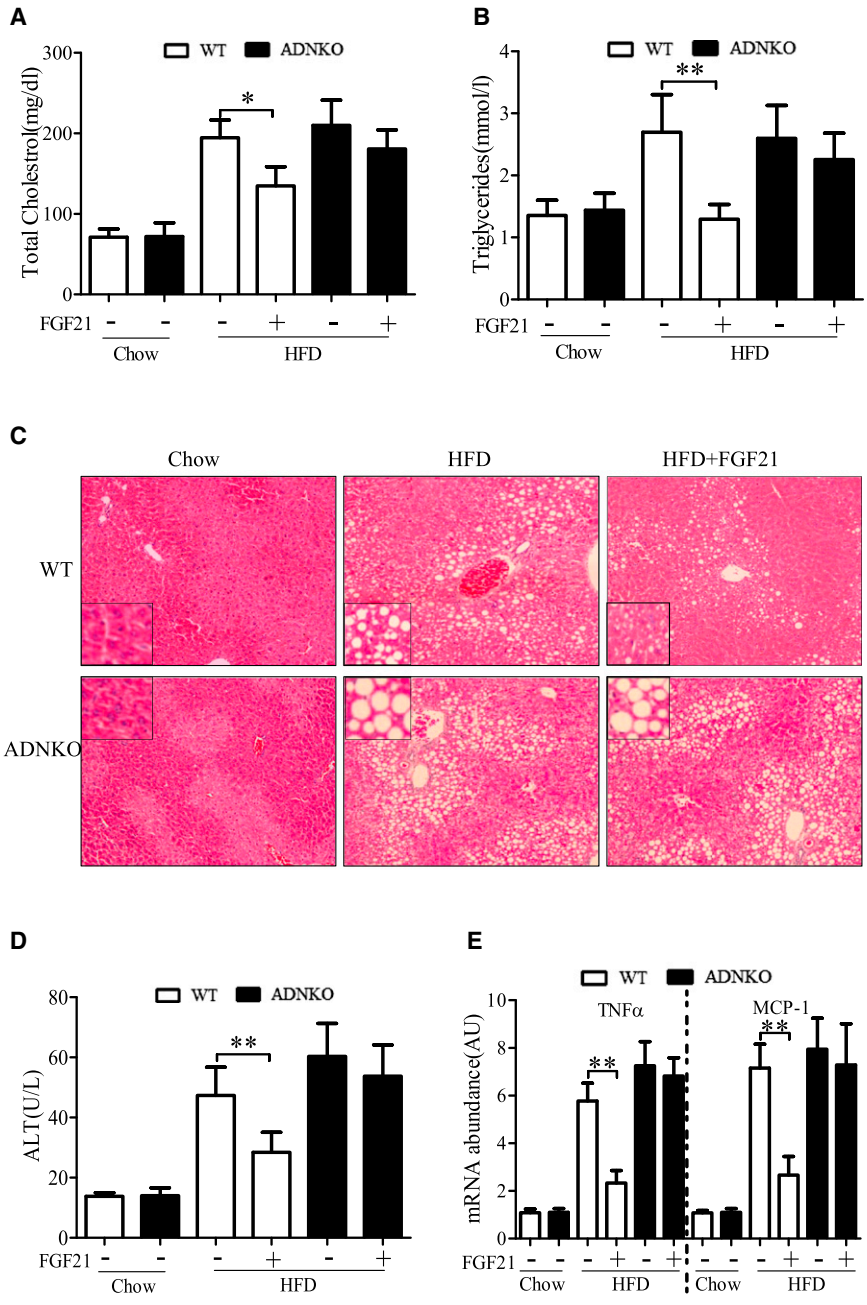

Figure 7. The Beneficial Effects of Chronic Administration with FGF21 on Dyslipidemia and Fatty Liver Are Compromised by Adiponectin Deficiency in Mice

ADNKO mice and WT mice on HFD were treated with rmFGF21 or vehicle control for 4 weeks as in Figure 6. Age- and sex-matched mice on Chow were used as controls.

(A) Serum levels of triglycerides.

(B) Serum levels of total cholesterol

(C) H&E staining of liver sections.

(D) Serum levels of ALT.

(E) The mRNA expression of TNF-α and MCP-1 as quantified by real-time PCR.

Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. $n = 6$ in each group. See also Figures S4 and S5 and Table S1.

et al., 2013) and β-klotho knockout mice (Ding et al., 2012) confirmed an obligatory role of adipocytes in FGF21-mediated metabolic homeostasis. However, it is still unclear how FGF21 actions in adipocytes lead to profound systemic changes in glucose and lipid metabolism. In the present study, we provide both in vitro and animal evidence showing that FGF21 induces the biosynthesis of adiponectin, which in turn acts in an endocrine manner to exert its effects on insulin sensitization and metabolic regulation in other major metabolic organs.

Adiponectin biosynthesis in adipocytes is tightly controlled at both the transcriptional and posttranslational levels (Wang et al., 2008; Wang et al., 2006). In obesity, adipocyte hypertrophy causes impaired expression and secretion of adiponectin, thereby leading to hypoadiponectinemia, which is an independent risk factor for insulin resistance and diabetes (Li et al., 2011). Therefore, therapeutic interventions that augment the production of endogenous adiponectin have been proposed as a promising treatment modality for obesity-related metabolic disorders (Li et al., 2011). In the present study, we showed that FGF21 increases serum adiponectin by enhancing both its gene transcription and protein secretion in adipocytes. FGF21 acutely increases adiponectin secretion in adipocytes and elevates its circulating concentrations in mice, both of which occur well before transcriptional activation of the adiponectin gene. On the other hand, the amplitude of elevation in adiponectin production by chronic infusion of FGF21, which is associated with increased levels of adiponectin gene expression and the protein abundance of several molecular chaperones involved in adiponectin oligomerization, is much higher than that by acute FGF21 injection. Therefore, it is likely that the acute effect of FGF21 is due to its ability in stimulating adiponectin release via a nongenomic mechanism, whereas chronic actions of FGF21 on sustained elevation of circulating adiponectin are attributable to both increased gene expression and protein secretion of adiponectin.

In obese subjects and type 2 diabetic patients, serum FGF21 levels are elevated (Zhang et al., 2008; Chen et al., 2011a), whereas serum adiponectin levels are decreased (Weyer et al., 2001; Lindsay et al., 2002). There are several possible mechanisms that could account for the paradoxical dissociation between serum levels of adiponectin and FGF21 in obesity and type 2 diabetes. First, FGF21 is not the only regulator of adiponectin production. Many factors that negatively regulate adiponectin expression and secretion, such as proinflammatory

cytokines, endoplasmic reticulum stress, and oxidative stress, are progressively increased in adipose tissues during the development of obesity (Fasshauer et al., 2003; Liu and Liu, 2010). Therefore, elevated FGF21 is not sufficient to counteract the effects of these negative regulators on adiponectin production under this circumstance. This assumption is supported by our observation that FGF21 KO mice are more susceptible to HFD-induced reduction in serum adiponectin as compared to WT controls. Second, FGF21 resistance in adipose tissues has been reported in obese animals (Fisher et al., 2010; Ge et al., 2011) and may be further aggravated by chronic inflammation with the progression of obesity (Díaz-Delfín et al., 2012). Therefore, it is likely that the ability of FGF21 to induce adipocyte production of adiponectin is diminished due to FGF21 resistance in obesity and type 2 diabetes.

A recent study has demonstrated a critical role of a positive feedback loop between FGF21 and PPARγ in adipocytes in maintaining systemic glucose homeostasis (Dutchak et al., 2012). The autocrine actions of FGF21 activate PPARγ, which in turn leads to further induction of FGF21 expression via transcriptional activation. Our present study demonstrated that both the acute and chronic effects of FGF21 on adiponectin production are mediated in part by PPARγ, a nuclear receptor that controls the gene transcription as well as the protein secretion of adiponectin (Wang et al., 2007; Iwaki et al., 2003). Furthermore, we found that the stimulatory effects of the PPARγ agonists TZDs on adiponectin production in both mouse adipocytes and human adipose tissue are mediated by augmented autocrine actions of FGF21. Taken together, these findings suggest that TZDs exert their antidiabetic actions by amplifying the autocrine signal of FGF21 in adipocytes, which consequently triggers the release of adiponectin into the bloodstream for alleviation of peripheral insulin resistance (Figure S3). In support of this notion, both FGF21 and ADNKO mice are refractory to the therapeutic benefits of TZDs on insulin sensitivity and glucose homeostasis (Dutchak et al., 2012; Nawrocki et al., 2006; Kubota et al., 2006).

FGF21 has been shown to exert multiple metabolic effects in the liver, including alleviation of diet-induced hepatic insulin resistance and hepatic steatosis (Xu et al., 2009b) and regulating hepatic gluconeogenesis and ketogenesis (Potthoff et al., 2009; Badman et al., 2007). However, whether liver is the direct target of FGF21 remains debatable (Potthoff et al., 2009; Fisher et al., 2011). Hepatocytes express β-klotho but not FGFR1, the latter of which is a major receptor isoform for FGF21 (Suzuki et al., 2008). In vivo administration of FGF21 enhances hepatic fatty acid oxidation and tricarboxylic acid cycle flux via inducing the expression of PGC1α and its downstream target genes (Potthoff et al., 2009). By contrast, direct incubation of either isolated mouse liver or primary cultures of rat hepatocytes with FGF21 has no such effects, implicating that FGF21 exerts these hepatic actions via an indirect mechanism that may involve other secretory factors. In line with these findings, our present study suggests adiponectin to be an obligatory mediator for these hepatic actions of FGF21. This notion is supported by our observation that the chronic effects of FGF21 on alleviation of diet-induced hepatic insulin resistance, steatosis, and liver injury are abolished in ADNKO mice. Indeed, hepatocytes are the direct target of adiponectin, where this adipokine inhibits fatty acid synthesis, enhances fatty acid oxidation, and increases insulin sensitivity (Berg et al., 2001; Xu et al., 2003). Likewise, the lack of responses to FGF21-mediated lipid depletion and insulin sensitization in skeletal muscle of ADNKO mice also suggests that the muscular effects of chronic FGF21 administration are also mediated by adiponectin, but not due to the direction action of FGF21 in this tissue.

While the metabolic effects of chronic FGF21 administration on liver and skeletal muscle are largely abolished by adiponectin deficiency, it is of interest to note that its activities in adipose tissue, including activation of ERK1/ERK2 and reduction of fat mass, remain intact. In adipocytes, FGF21-mediated activation of ERK1/ERK2 leads to the induction of glucose transporter-1 expression, thereby enhancing insulin-independent glucose uptake (Ge et al., 2011; Kharitonenkov et al., 2005). Since pharmacological inhibition of ERK1/ERK2 has no obvious effect on FGF21-induced adiponectin production in adipocytes, this kinase does not appear to be an important player in mediating the systemic effects of FGF21 on glucose metabolism and insulin sensitivity. FGF21-mediated fat mass loss has been attributed to its ability in enhancing energy expenditure (Coskun et al., 2008), possibly resultant from increased transdifferentiation of white to brown adipocytes (Potthoff et al., 2012). While our present study showed a comparable degree of FGF21-mediated reduction of diet-induced obesity between ADNKO mice and WT controls, such a beneficial effect of FGF21 is compromised in mice with adipose tissue selective depletion of either FGFR1 or β-klotho (Adams et al., 2013; Yang et al., 2012). Taken together, these findings suggest that the reduction of fat mass by FGF21 is a consequence of its direct actions in adipocytes, independent of adiponectin. Indeed, unlike FGF21 transgenic mice resistant to diet-induced obesity (Inagaki et al., 2007), transgenic expression of adiponectin causes morbid obesity despite a healthy metabolic profile (Kim et al., 2007), highlighting the differences between these two factors in regulating adiposity.

Another notable observation of the present study is that adiponectin deficiency leads to a compromised effect of FGF21 on suppression of HFD-induced hypertriglyceridemia, but not hypercholesterolemia. In line with our findings, a recent study on lipodystrophic mice demonstrated that adipose tissue is obligatory in mediating the triglyceride-lowering effects of FGF21 but is not required for its cholesterol-lowering activities (Véniant et al., 2012). Therefore, it is likely that the beneficial effects of FGF21 on hypertriglyceridemia are attributable to its ability in facilitating fatty acid combustion in both liver and skeletal muscle, whereas FGF21-mediated reduction of hypercholesterolemia is independent of its actions on adipocytes and does not require adiponectin. In support of this notion, adiponectin has been reported to decrease circulating levels of triglycerides, but not cholesterol (Yamauchi et al., 2001).

In summary, our present study has demonstrated a central role of adiponectin in conferring the therapeutic benefits of FGF21 in improving obesity-induced hyperglycemia, hypertriglyceridemia, and peripheral insulin resistance. These findings provide a molecular mechanism to explain how FGF21, via its actions in adipocytes, controls systemic glucose and lipid homeostasis and insulin sensitivity in the major metabolic organs.

EXPERIMENTAL PROCEDURES

Animals

Male FGF21 KO mice (Hotta et al., 2009), ADNKO mice (Ma et al., 2002), PPARγ heterozygous-deficient mice ($Pparg^{tm3Yba}$/$Pparg^{+}$, Jackson laboratory, $PPAR^{+/-}$), leptin $receptor^{-/-}$ (*db*/*db*) mice and leptin $receptor^{-/-}$/$adiponectin^{-/-}$ double knockout (DKO) mice (Zhou et al., 2008) with a C57BL/6J background, and WT controls with the same genetic backgroup were used for this study. These mice were fed with either standard chow or an HFD diet (D12451, Research Diet, New Brunswick, NJ) containing 45% fat, 20% protein, and 35% carbohydrate (kcal%). All animals were kept under 12 hr light-dark cycles at 22°C –24°C, with free access to water. Glucose tolerance and insulin tolerance tests were conducted as we previously described (Xu et al., 2009a). All of the experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

Metabolic Effects of rmFGF21

rmFGF21 and rabbit anti-human FGF21 IgG were prepared as described previously (Yu et al., 2011; Ge et al., 2011). For the acute studies, 12-week-old mice were injected intraperitoneally with either rmFGF21 (2 mg/kg) or saline as vehicle control. Blood samples were collected at various time points after injection for measurement of glucose, triglycerides, and cholesterol as described (Xu et al., 2009a). For chronic studies, 8-week-old ADNKO mice or WT controls which had been on HFD for 4 weeks were implanted with an Alzet osmotic pump as described previously (Xu et al., 2003), to continuously deliver rmFGF21 for 4 weeks (0.5 mg/kg per day). Glucose and insulin tolerance tests were performed 2 and 3 weeks after treatment, respectively. Total fat and lean mass was quantified with a Brucker Minispec Body composition analyzer. Mice were sacrificed after 4 weeks of treatments for collection of various tissues for further biochemical and histological evaluations.

Differentiation of Mouse Adipocytes

Stromal vascular cells were isolated from epididymal fat pads of 8-week-old FGF21 KO mice or WT controls by collagenase digestion and centrifugation and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Differentiation was initiated as described (Xu et al., 2009a). Ten days after differentiation, more than 80% of cells became mature adipocytes filled with multiple lipid droplets as assessed by oil red O staining.

Pulse-Chase Experiment with ^{35}S-Labeled Methionine and Cysteine

Pulse-chase experiment with ^{35}S-labeled methionine and cysteine were performed as described (Xu et al., 2009a). In brief, mouse adipocytes were starved in methionine- and cysteine-free DMEM for 1 hr and were then incubated with the same fresh medium plus 50 μCi/ml [35S]methionine and [35S] cysteine (Redivue Pro-mix L-[35S]; GE Healthcare, Piscataway, NJ) for another 1 hr. The medium was replaced subsequently with cold DMEM with a 20-fold excess of methionine and cysteine for different time periods. Both the culture medium and cells were harvested for immunoprecipitation with affinity-purified rabbit anti-mouse adiponectin IgG as described (Xu et al., 2005). The immunoprecipitated complexes were eluted by incubation with a SDS-PAGE loading buffer. The eluted samples were separated by 15% gradient SDS-PAGE and then analyzed by phosphorimaging (BAS 2000; Fujifilm, Tokyo, Japan).

Ex Vivo Studies with Human Adipose Tissue Explants

Human subcutaneous fat tissues were obtained from five healthy premenopausal Chinese women undergoing abdominal surgery for benign gynecological conditions and minced into small pieces. The fat explants were cultured in DMEM medium with 0.5% BSA for 30 min and were then treated with rosiglitazone and in the presence of 2 μg/ml rabbit anti-human FGF21 antibody (Yu et al., 2011) or nonimmune IgG for 24 hr. The conditioned medium and cells were collected to quantify the protein secretion and mRNA expression of adiponectin. All subjects gave informed consent, and all the procedures were approved by the local ethics committee.

Biochemical and Immunological Assays

Serum ALT activities were determined with a commercial kit from Sigma-Aldrich (St. Louis, MO). Serum levels of insulin, total adiponectin, and FGF21 were quantified with immunoassays from Antibody and Immunoassay Services at University of Hong Kong. Different oligomeric forms of adiponectin in serum samples were analyzed by gel filtration as described (Xu et al., 2005). Serum FFA and β-hydroxybutyrate concentrations were measured with the Free Fatty Acids, Half Micro Test (Roche) and β-hydroxybutyrate Assay Kit (Abcam), respectively.

Immunoblotting and Histological Analysis

Proteins were extracted from liver tissues in the presence of protease inhibitors cocktail (Roche Applied Science), resolved by SDS-PAGE, and then transferred to PVDF membranes. The membranes were then probed with primary antibodies against Ero1-Lα (Santa Cruz Biotechnology), phospho-Akt, Akt, phosphor-GSK3β, GSK3β (Cell Signaling Technology), and DsbA-L (Abcam) followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized with enhanced chemiluminescence reagents (GE Healthcare, Uppsala, Sweden) and quantified with the NIH Image J software.

Liver specimens were fixed in 10% formalin solution (Sigma) and embedded in paraffin. Liver tissue sections were stained with H&E via standard procedures. All slides were examined under Olympus biological microscope BX41, and images were captured with an Olympus DP72 color digital camera.

RNA Extraction and Real-Time PCR

Total RNA was extracted from liver or adipose tissues with TRIzol reagent (Invitrogen), and complementary DNA was synthesized from 0.5 μg total RNA by reverse transcription with an ImProm-II reverse transcription kit (Promega) with random hexamer primers. Quantitative real-time PCR was performed through the use of the SYBR Green QPCR system (QIAGEN) with specific primers (Table S2). The PCR reactions were performed with an Applied Biosystems Prism 7000 sequence detection system. The level of target gene expression was normalized against the GAPDH gene.

Statistical Analysis

Experiments were performed routinely with five to six mice per group. All analyses were performed with Statistical Package for Social Sciences version 14.0 (SPSS, Chicago, IL). Data were expressed as mean ± SEM. Comparison between groups was performed via ANOVA followed by Bonferroni correction in the case of multiple comparisons. In all statistical comparisons, a p value < 0.05 was used to indicate a statistically significance difference.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.04.005.

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