FGF21 does not require adipocyte AMP-activated protein kinase (AMPK) or the phosphorylation of acetyl-CoA carboxylase (ACC) to mediate improvements in whole-body glucose homeostasis

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Keywords: FGF21; AMPK; ACC; Adipocyte; Brown fat; Obesity; Diabetes

Objective: Fibroblast growth factor 21 (FGF21) shows great potential for the treatment of obesity and type 2 diabetes, as its long-acting analogue reduces body weight and improves lipid profiles in participants in clinical studies; however, the intracellular mechanisms mediating these effects are poorly understood. AMP-activated protein kinase (AMPK) is an important energy sensor of the cell and a molecular target for anti-diabetic medications. This work examined the role of AMPK in mediating the glucose and lipid-lowering effects of FGF21.

Methods: Inducible adipocyte AMPK β1/2 knockout mice (β1/2AKO) and littermate controls were fed a high fat diet (HFD) and treated with native FGF21 or saline for two weeks. Additionally, HFD-fed mice with knock-in mutations on the AMPK phosphorylation sites of acetyl-CoA carboxylase (ACC1 and ACC2 (DKI mice)) along with wild-type (WT) controls received long-acting FGF21 for two weeks.

Results: Consistent with previous studies, FGF21 treatment significantly reduced body weight, adiposity, and liver lipids in HFD fed mice. To add, FGF21 improved circulating lipids, glycemic control, and insulin sensitivity. These effects were independent of adipocyte AMPK and were not associated with changes in brown of white (WAT) and brown adipose tissue (BAT). Lastly, we assessed whether FGF21 exerted its effects through the AMPK/ACC axis, which is critical in the therapeutic benefits of the anti-diabetic medication metformin. ACC DKI mice had improved glucose and insulin tolerance and a reduction in body weight, body fat and hepatic steatosis similar to WT mice in response to FGF21 administration.

Conclusions: These data illustrate that the metabolic improvements upon FGF21 administration are independent of adipocyte AMPK, and do not require the inhibitory action of AMPK on ACC. This is in contrast to the anti-diabetic medication metformin and suggests that the treatment of obesity and diabetes with the combination of FGF21 and AMPK activators merits consideration.

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Acknowledgments: ACC, acetyl-CoA carboxylase; ACC DIK, ACC1-S79A and ACC2-S212A double knock-in; AKT, protein kinase B; AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; COX, cytochrome b oxidase; CreER2, Cre recombinase — estrogen receptor T2; CNS, central nervous system; DAG, diacylglycerol; FFA, free fatty acid; FGF21, fibroblast growth factor 21; FGRF1c, fibroblast growth factor receptor 1c; GGT, glucose tolerance test; gWAT, gonadal white adipose tissue; H&E, hematoxylin and eosin; HFD, high fat diet; iβ1/2AKO, inducible AMPK β1/2 adipocyte knockout; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; KLB, beta klotho; RER, respiratory exchange ratio; mTORC1, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; TAG, triacylglycerol; UCP1, uncoupling protein 1; WT, white adipose tissue; WT, wildtype

Received March 1, 2017 • Revision received March 28, 2017 • Accepted April 2, 2017 • Available online xxx

http://dx.doi.org/10.1016/j.molmet.2017.04.001
1. INTRODUCTION

Initially identified as a hormone secreted by the liver that could potentially stimulate glucose uptake in adipocytes [1], fibroblast growth factor 21 (FGF21) is an endocrine factor that exerts potent anti-obesity and anti-diabetic effects in rodents and non-human primates [2–8]. Furthermore, long-acting FGF21 analogues in phase 1 clinical trials decrease body weight and improve the lipid profile of patients with type 2 diabetes [6,9]. In pre-clinical rodent models, FGF21 administration increases energy expenditure and improves glucose and lipid homeostasis [2]. These effects have been related to increases in glucose uptake and triglyceride clearance in white (WAT) and brown adipose tissue (BAT) [10] and require the expression of the FGF21 receptor, fibroblast growth factor receptor 1c (FGFR1c), and cofactor beta klotho (KLB) [11,12]. Findings from several FGF21 studies have also shown increases in the browning of WAT; a process whereby WAT

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RT-qPCR was performed to determine mRNA expression of browning and mitochondrial markers as previously described [36]. Briefly, RNA was isolated using TRIzol reagent (Invitrogen; CA, USA) and applied to columns (RNeasy kit, Qiagen; CA, USA) for purification. All Taqman primers were purchased from Invitrogen [23] (Hadh, Mm00492535_m1; Cpt1b, Mm00487191_g1; Pparγ1a, Mm01208835_m1; Ppara, Mm00409399_m1; Ucp1, Mm01244661_m1; Ppia, Mm02342430_g1), and relative gene expression was calculated using the comparative Ct (ΔΔCT) method. Values were normalized to the housekeeping gene Ppia and subsequently expressed as relative to control saline.

2.8. Statistical analyses
Results were analyzed by two-way ANOVA using GraphPad Prism software. Repeated measures two-way ANOVA was used for GTT and ITT data. All values are presented as mean ± SEM and significance was accepted at p ≤ 0.05.

3. RESULTS

3.1. FGF21 increases energy expenditure, reduces adiposity, and improves blood parameters independent of adipocyte AMPK
To examine the role of adipocyte AMPK in mediating the metabolic effects of FGF21, double floxed AMPK β1 and β2 mice with or without the Adiponectin Cre recombinase — estrogen receptor T2 (CreERT2) transgene were subjected to HFD treatment for 10 weeks followed by tamoxifen-induced deletion of AMPK (Figure 1A). We evaluated the efficiency of deletion by determining phosphorylation of ACC, the most sensitive method for detecting cellular AMPK activity, and found no detectable phosphorylation of ACC in BAT and iWAT of mice deficient for AMPK (Supplemental Figure 1). Following three weeks to allow for the appropriate genetic recombination and recovery, mice treated with native FGF21 for two weeks showed increased oxygen consumption and energy expenditure (Figure 1B–D) and lowered respiratory exchange ratio (R.E.R.) (Figure 1E) in both control and mice deficient for AMPK in adipocytes. The increase in metabolic activity by FGF21 was similar when the data were normalized to lean body mass (Figure 1B vs. C) and was not due to differences in food intake and physical activity, as these parameters were unchanged upon FGF21 administration (Figure 1F and G). Concomitant with greater metabolic activity, FGF21 treatment reduced body weight (Figure 1H) and adiposity (Figure 1I) in both genotypes. Overall, these data indicate that FGF21 does not require adipocyte AMPK to mediate an increase in energy expenditure and a reduction in body fat.

Adiponectin has been suggested to be an important mediator for the insulin sensitizing effects of FGF21 [3,37] and is an activator of AMPK [38]. Indeed, FGF21 administration increases circulating adiponectin in humans [6,9]. FGF21 administration caused a modest trend of increased circulating adiponectin levels in control (p = 0.068) (Figure 2A) but not in β1β2KO mice; however, serum insulin levels were dramatically reduced in both genotypes (Figure 2B). Despite the much lower insulin levels, WT and β1β2KO mice treated with FGF21 had lower fed (Figure 2C) and fasted blood glucose levels (Figure 2D). FGF21 treatment also lowered plasma free fatty acid (FFA) and triacylglycerol (TAG) levels in both genotypes to a similar extent (Figure 2E and F). Overall, these data indicate that FGF21 lowers serum insulin, blood glucose, and plasma TAGs and FFAs independently of adipocyte AMPK.

3.2. FGF21 improves insulin sensitivity and promotes glucose uptake in BAT independently of adipocyte AMPK
Consistent with lower blood glucose and plasma insulin, FGF21 improved insulin-mediated glucose disposal in both control and β1β2KO mice (Figure 3A and B). To determine which tissues were mediating the improvements in insulin-mediated glucose uptake, we assessed 2-deoxyglucose uptake following an intraperitoneal injection.
of insulin. FGF21 increased glucose uptake in BAT (Figure 3C), but this effect was not significant in iWAT (Figure 3D). Interestingly, FGF21 had an overall effect of lowering glucose uptake in gonadal WAT (gWAT), liver and quadriceps muscle (Figure 3E–G). To determine if FGF21 treatment was associated with improvements in adipose tissue insulin signaling, and whether there were differences in iβ1β2AKO mice, we examined AKT phosphorylation status in BAT and iWAT. FGF21 treatment increased phosphorylation of AKT in both BAT (Figure 3H and I) and iWAT (Figure 3J) following an acute injection of insulin. These data indicate that FGF21 treatment improves BAT insulin action and that these effects do not require AMPK.

3.3. FGF21 reduces hepatic steatosis without increasing WAT browning
FGF21 reduces non-alcoholic fatty liver disease (NAFLD) [2,5] while inducible deletion of adipocyte AMPK prior to HFD treatment promotes NAFLD [23]. Consistent with previous reports [2,29], FGF21 treatment resulted in lower hepatic lipid content, as indicated by lower lipid-laden white area present within cells in hematoxylin and eosin (H&E) stains (Figure 4A), which was further confirmed by a reduction in liver diacylglycerol (DAG) (Figure 4B) and TAG (Figure 4C) levels. In contrast to DAG and TAG levels, liver ceramide levels were increased with FGF21 treatment (Figure 4D).
FGF21 has been associated with improvements in BAT function and increased WAT browning; however, the role of UCP1 and WAT browning in mediating the metabolic improvements induced by pharmacological administration of FGF21 are somewhat dichotomous [13,15,16]. FGF21 treatment reduced both BAT and iWAT mass (Figure 5A and B, respectively) and reduced the presence of lipid in BAT, as indicated by H&E staining (Figure 5C). FGF21 treatment had a modest overall effect on increasing UCP1 and MT-CO2 mRNA levels in BAT of control and iβ1β2AKO mice (Figure 5D), but not in iWAT (Figure 5F). Consistent with an increase in MT-CO2, FGF21 increased mitochondrial cytochrome c oxidase (COX) activity in the BAT of both control and iβ1β2AKO mice (Figure 5E). FGF21 increased Pparγc1a mRNA levels in iWAT, but the expression of other browning markers was unaffected (Figure 5F). FGF21 treatment did not affect UCP1 protein levels in BAT, while there was an overall effect for lower UCP1 levels in the BAT of iβ1β2AKO mice (Figure 5G and H). The expression of UCP1 protein in iWAT, while highly variable, was unaffected by FGF21 treatment in either Control or iβ1β2AKO mice (Figure 5G and H).

3.4. FGF21-mediated improvements in glucose homeostasis, insulin sensitivity and hepatic lipid content are independent of phosphorylation of ACC by AMPK

The accumulation of hepatic lipids is thought to be critical in liver insulin resistance and causative in the dysregulation of whole-body glucose homeostasis [39]. One of the molecular mechanisms for hepatic lipid accumulation is the process of de novo lipogenesis. FGF21 has been shown to suppress hepatic glucose output [28] and de novo lipogenesis, effects that were associated with the activation of AMPK [2]. To examine whether the metabolic improvements resulting from FGF21 administration are due to AMPK’s inhibitory
action on ACC — a key rate-limiting enzyme in the lipogenesis pathway and regulator of hepatic fat oxidation — we utilized a previously described knock-in mutation mouse model for ACC where AMPK lacks the ability to phosphorylate and inactivate ACC [31]. In addition, we utilized a long-acting form of FGF21 (PF-05231023) that demonstrated efficacy in humans [6]. Consistent with previous studies [2,5] and the current data in ij1/2AKO mice, FGF21 significantly improved glucose tolerance (Figure 6A and B) and insulin sensitivity (Figure 6C and D). However, these effects were independent of the ability of AMPK to phosphorylate and inactivate ACC. Additionally, FGF21 administration led to reduced body weight (Figure 6E), adiposity (Figure 6F), and lowered fed glucose (Figure 6G) and insulin crosstolerance in both C57L/J and 129SvJ backgrounds. Together, these results identify FGF21 as a potential therapeutic target for the treatment of type 2 diabetes and nonalcoholic fatty liver disease.

Figure 3: Control and ij1/2AKO mice treated with native FGF21 for two weeks have improved insulin sensitivity. A and B: insulin tolerance test (ITT: 1 U/kg) (A) and area under the curve (AUC) (B) of control and ij1/2AKO mice treated with native FGF21 on indicated diet (n = 4–7 per group). C–G: tissue glucose uptake measured using 2-DG of BAT (C), iWAT (D), gWAT (E), liver (F), and quadriceps muscle (G) in control and ij1/2AKO mice treated with native FGF21 (n = 4–7 per group). H–J: representative total and phospho (S473) AKT immunoblotting (H) with quantification in BAT (i; n = 4–7 per group) and iWAT (j; n = 3–7 per group) of 10-week HFD and 2-week treated (saline or native FGF21) control and ij1/2AKO mice. Data are means ± SEM with ||| p < 0.001, || p < 0.01, and p < 0.05 denoting a general treatment effect as determined by a two-way ANOVA (A: two-way repeated measures ANOVA).
Figure 4: Treatment with native FGF21 for two weeks reduces liver lipids in mice fed a high fat diet (45%) for 10 weeks. (A) representative H&E liver stains (20x) of control and i(1)2AKO mice treated with saline or native FGF21. B and C: Liver levels of diacylglycerol (B) and triacylglycerol (C) as measured by mass spec (n = 4–7 per group). (D) Liver ceramides as measured by mass spec (n = 4–7 per group). Data are means ± SEM with †p < 0.05 denoting a general genotype effect as determined by a two-way ANOVA.

Figure 5: The beneficial effects of native FGF21 in control and i(1)2AKO mice are not driven by the upregulation of a thermogenic program in vivo. A and B: tissue weights of BAT (A) and iWAT (B) of control and i(1)2AKO mice treated with saline or native FGF21 for 14 days (n = 4–7 per group). (C) representative H&E liver stains (20x) of BAT from control and i(1)2AKO mice fed a high fat diet (45%) for 10 weeks and treated with saline or native FGF21 for 14 days. D and E: mRNA expression of mitochondrial and browning markers (Hadh, MT-CO2, Cox8b, Cpt1b, Pparc1α, Pparz, Cidea, Ucp1) in BAT (D) and iWAT (E) (n = 4–7 per group). (F) mitochondrial COX activity in whole tissue BAT lysates from control and i(1)2AKO mice treated with FGF21 (n = 4–7 per group). G–H: representative UCP1 and β-tubulin immunoblotting (G) with quantification in BAT (H) and iWAT (I) of high fat diet-fed and two week treated (saline or native FGF21) control and i(1)2AKO mice (n = 4–7 per group). Data are means ± SEM with †† †p < 0.001, †† †p < 0.01, †p < 0.05 denoting a general treatment effect and *p < 0.05 denoting a general genotype effect as determined by a two-way ANOVA.

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fasted-state (Figure 6H) blood glucose levels, irrespective of genotype. Lastly, the administration of long-acting FGF21 substantially lowered hepatic lipid content, marked by the reduced lipid containing area within H&E stained liver sections (Figure 6I) and reduced liver triacylglycerol levels (Figure 6J).

4. DISCUSSION

In the current study, we examined whether pharmacological administration of FGF21 requires adipocyte AMPK to mediate its beneficial effects on metabolic homeostasis. Our previous work demonstrated that adipocyte AMPK was necessary to maintain BAT function, mediate increases in WAT browning and protect against the development of hepatic steatosis in rodents on a high fat diet [23]. Our current results demonstrate that FGF21 administration does not require adipocyte AMPK to mediate improvements in glucose homeostasis, lipid homeostasis, insulin sensitivity, and reductions in body weight in vivo. Furthermore, we tested whether AMPK’s inhibitory phosphorylation of ACC mediated the metabolic benefits of FGF21 administration and showed no genotype differences in the treated groups.

In mice, FGF21-mediated improvements in metabolic homeostasis have also been associated with the browning of WAT [2,14]. We found that FGF21 treatment increased energy expenditure, reduced body fat mass, and improved insulin sensitivity without increases in BAT or iWAT UCP1 protein levels. Indeed, as previously reported [23], we found that BAT UCP1 levels were lower in mice deficient for adipocyte AMPK, yet FGF21 improved metabolic homeostasis in these mice. FGF21 increased mitochondrial COX activity in both genotypes, which may explain how FGF21 might be mediating its effects independently of adipocyte AMPK, possibly via UCP1-independent mechanisms (see Figure 6).

Figure 6: Long-acting (PF-05231023) FGF21-mediated improvements in insulin sensitivity and hepatic lipid content are not due to AMPK’s suppression of ACC. A–D: glucose tolerance test (ITT; 1 g/kg) (A) with area under the curve (AUC) (B) and insulin tolerance test (ITT; 1 U/kg) (C) with area under the curve (D) of wildtype and ACC DKI fed a 45% high fat diet for 10 weeks and treated with saline or long-acting FGF21 for two weeks (n = 4 per group). Change in body weights (E) and in fat percent (F) in wildtype and ACC DKI mice treated with saline or PF-05231023 (n = 8 per group). Blood glucose levels in the fed (G) and 12-hr fasted (H) state of wildtype and ACC DKI mice treated with saline or long-acting FGF21 for two weeks (n = 8 per group). I and J: representative H&E stained liver sections (20x) (I) and levels of triacylglycerol (J) in the liver of wildtype and ACC DKI mice treated with saline or long-acting FGF21 for 2 weeks (n = 8 per group). Data are means ± SEM with **p < 0.001, ***p < 0.001, ****p < 0.001, †††p < 0.001, ††††p < 0.001, †††††p < 0.001, ††††††p < 0.001 denoting a general genotype effect as determined by a two-way ANOVA (A and C: two-way repeated measures ANOVA).

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below). We did not observe changes in overall WAT browning, as measured by gene expression analyses and UCP1 protein levels, although FGF21 treatment did increase Ppargc1a mRNA levels, which could still be involved in mediating the effects of FGF21 [18,40] in this model.

We found that FGF21 increased glucose uptake in BAT and lowered uptake in the liver and quadriceps muscle, effects which were independent of adipocyte AMPK. Since we did not perform the hyperinsulinemic-euglycemic clamp technique, we cannot rule out the potential effects of FGF21 on suppressing hepatic glucose output [2,28]. FGF21 also increased tissue insulin action in BAT and iWAT independently of adipocyte AMPK, however, FGF21 did not significantly increase tissue glucose uptake in iWAT. These data suggest that the target tissue for the glucose lowering effects of FGF21 is likely BAT, although this does not require increased BAT UCP1 protein levels or AMPK. These data could explain the lack of glucose lowering in humans since the subjects recruited for the clinical studies were not measured for the presence of functional BAT. An alternate pathway is that FGF21 might promote UCP1-independent thermogenic mechanisms to mediate some of its beneficial metabolic effects. Recently, a phospho-proteomic approach to identify the signaling pathways activated downstream of FGF21 in adipocytes uncovered the metabolic sensor mammalian target of rapamycin (mTORC1) as an important mediator of the effects of FGF21 in vitro [41]. mTORC1 was required for FGF21-mediated increase in UCP1 mRNA, adiponectin secretion, and glucose uptake [41]. Future work should determine whether FGF21 requires adipocyte mTORC1 to mediate improvements in whole-body metabolism.

FGF21 likely improves hepatic steatosis and liver insulin action by reducing lipogenesis and enhancing lipid oxidation [2,27,29,30]. As metabolic improvements upon FGF21 administration are associated with reduced expression of ACC1 and ACC2 in liver [27], we used previously described knock-in mutation mice (ACC DKI) to test whether AMPK’s inhibitory phosphorylation of ACC (ACC1-S79 and ACC2-S212) mediated the metabolic benefits of FGF21 administration. When ACC is phosphorylated by AMPK, as occurs with metformin treatment, malonyl-CoA levels are reduced, leading to the suppression of de novo lipogenesis and the reduction of hepatic lipid content and insulin resistance [31,42]. However, we observed no differences between the wildtype and ACC DKI mice in response to FGF21 administration over two weeks. These data indicate that FGF21 does not share a similar mechanism to metformin for the amelioration of lipid content and insulin sensitivity [31].

In conclusion, we report that FGF21 improves metabolic homeostasis in HFD-fed mice, effects that are independent of adipocyte AMPK or its downstream substrate ACC, and which were not associated with increases in BAT or WAT browning. These data suggest that combinatorial treatment of obesity and diabetes with FGF21 and AMPK activators [31,43] may have enhanced efficacy since they function through different signaling pathways. Future work is required to determine whether FGF21 mediates its effects via UCP1-independent futile cycling pathways, such as greater lipid turnover [44] or a creatine substrate cycle [45].

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Dr. Thomas J. Hawke for allowing access to the microscope. We thank Dr. Bruce E. Kemp for providing the [11]32 mice and the ACC1/2 DKO mice. This study was supported by a grant from the Canadian Diabetes Association (G.R.S.). E.P.M. was a Canadian Diabetes Association Post-doctoral research fellow. E.M.D. was a recipient of an Ontario Graduate Scholarship and a Queen Elizabeth II Graduate Scholarship in Science and Technology. A.M.F. was funded by the Danish Diabetes Academy, supported by the Novo Nordisk Foundation. G.R.S. is a Canada Research Chair in Metabolism and Obesity and the J. Bruce Duncan Chair in Metabolic Diseases.

**CONFLICT OF INTEREST**

The authors declare no competing financial interests.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.04.001.

**REFERENCES**


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