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Inhibition of lipolysis may contribute to the acute regulation of plasma FFA and glucose by FGF21 in *ob*/*ob* mice

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ABSTRACT

FGF21 is a unique member of the fibroblast growth factors (FGFs) and a novel hormone that regulates glucose, lipid, and energy homeostasis. The beneficial effects of FGF21 reported thus far have mostly been from chronic treatments. In order to better understand the mechanism for FGF21 action, we evaluated the acute effects of FGF21 in vivo and in vitro. Here we report that a single injection of FGF21 acutely reduced plasma free fatty acid levels similar to its acute effects on plasma glucose in *ob*/*ob* mice. In vitro, FGF21 inhibited lipolysis in adipocytes during a short treatment and reduced total lipase activity. These results demonstrate the potential importance of adipocyte lipolysis to the observed acute improvements in plasma parameters.

1. Introduction

FGF21 is a unique member of fibroblast growth factors (FGFs) and has emerged as an important metabolic hormone that is involved in the regulation of energy and glucose homeostasis [1,2]. The chronic effects of FGF21 on various metabolic parameters in several different disease models have been very impressive. FGF21 transgenic mice showed resistance to the effects of a high-fat high-calorie diet [1]. Chronic treatment with recombinant FGF21 has been shown to significantly reduce blood glucose levels without hypoglycemia in *ob*/*ob*, *db*/*db* and diet-induced-obesity (DIO) mice [1,3,4]. Improvements in glucose disposal and plasma lipid profiles have also been observed. In addition, all these benefits are accompanied by a significant weight loss due to increased energy expenditure without observed reduction in food intake. Furthermore, a recent study showed that FGF21 treatment improved glucose, insulin and lipid profiles, and reduced body weight of diabetic rhesus monkeys [5]. Therefore, FGF21 has the potential to be a novel therapy for the treatment of diabetes and obesity.

Recent progress has been made in the elucidation of receptor complexes and cellular functions for FGF21. In vitro studies suggest that in addition to the tyrosine kinase FGF receptors (FGFRs), β-Klotho (a single-pass transmembrane glycoprotein) is required as a co-receptor for FGF21 signaling on the cell surface [6–8]. Expression patterns of receptors may determine the target tissue specificity for FGF21 function. β-Klotho is predominantly expressed in metabolic organs including adipose tissue, liver, and pancreas, and direct effects of FGF21 on these cells have been demonstrated [3,6]. In adipocytes, FGF21 has been shown to stimulate glucose uptake independent of insulin, but these effects required overnight treatment with the protein and were dependent upon the increased transcription of glucose transporter GLUT1 [1]. However, these previous in vitro and in vivo studies have focused primarily on chronic or sub-chronic effects of FGF21. Studying acute responses will facilitate the understanding of the mechanism of action and to separate direct from secondary effects of FGF21 treatment.

In the current study, we sought to determine if FGF21 could acutely regulate lipid parameters. Here we report that a single intraperitoneal (i.p.) injection of FGF21 acutely lowered plasma glucose and free fatty acid (FFA) levels. In vitro, a short FGF21 treatment also inhibited lipolysis in adipocytes.

Abbreviations: FGF, fibroblast growth factor; FFA, free fatty acid

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2. Materials and methods

2.1. FGF21 protein

Recombinant human FGF21 protein (amino acids 29–208 without signal peptide) was expressed and purified from an *Escherichia coli* strain as previously described [3].

2.2. Glucose uptake assay

3T3L1 preadipocytes (ATCC CL-173) were cultured and induced to differentiate as follows: cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal FBS and 1% Pen-Strep at a density of 25 000 cells per well on 96-well Cytostar-T scintillating microplates (GE Healthcare). Two days after reaching confluence, differentiation was induced by adding 250 nM dexamethasone (Sigma), 500 μM isobutylmethylxanthine (Sigma) and 1 μg/ml insulin (Sigma) for 2 days. The cells were then cultured in DMEM with 10% FBS, 1% Pen-Strep and 1 μg/ml insulin for 2 days, and then without insulin for an additional 3 days. Differentiated adipocytes were washed once with DMEM containing 1% FBS and 1% Pen-Strep. Treatments were added to the adipocytes at the indicated concentrations and incubated for 4–72 h. The cells were then washed once with glucose-free DMEM (Invitrogen) containing 0.1% fatty-acid free BSA, and treatments were added to the cells at the indicated concentrations in the same medium for 3 h. The cells were then washed twice with Krebs–Ringer Phosphate Buffer (KRP) composed of 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 15 mM HEPES (pH 7.4), with 0.1% fatty-acid free BSA. 2-Deoxyglucose-1-^{3}H (Sigma) was added to the cells at a concentration of 0.2 μCi per well and incubated for an additional 1 h at 37 °C. Cytochalasin B (50 μM) (Sigma) was added to the cells to terminate the reaction and deoxyglucose uptake was measured on a Wallac MicroBeta (Perkin Elmer). Non-specific deoxyglucose uptake was measured in the presence of 50 μM cytochalasin B and subtracted from each sample to obtain specific uptake.

2.3. FGF21 treatment in mice

Aged matched C57Bl/6 or *ob*/*ob* mice were used in all the studies. Purified FGF21 or vehicle (PBS) were intraperitoneally (i.p.) injected into mice at stated concentrations. After indicated time, blood was collected by tail bleeding and blood glucose was measured with a glucose meter. Plasma FFA levels were measured using the Wako HR Series FFA-HR [2] kit following the manufacturer's instruction.

2.4. Lipolysis assay in differentiated 3T3L1 cells and primary adipocytes isolated from mice

3T3L1 cells (ATCC CL-173) were cultured and differentiated, and primary adipocytes were isolated from mice following protocols as previously described [9]. Adipocytes were then treated with FGF21 in KRH buffer for indicated times. Glycerol released from lipolysis during treatment was measured by Free Glycerol Reagent (Sigma F6428), and cells were used to measure the total lipase activity.

2.5. Total lipase activity measurement

Cells were treated with lysis buffer (0.25 M sucrose, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA). Cell lysate was centrifuged at 10000×*g* for 30 min at 4 °C. The upper level fat cake was removed and the clear supernatant was used to measure the total lipase activity. The lipase activity was measured using a fluorescent substrate from a lipase assay kit according to the manufacturer's instructions (Roar Biomedicals, RB- LPL2).

3. Results

3.1. Single injection of FGF21 into ob/ob mice acutely reduced blood glucose levels

Chronic studies have shown that FGF21 treatment for over 3 weeks, delivered by daily s.c. injection or by miniosmotic pump, lowered blood glucose levels and corrected obesity in *ob*/*ob* or DIO mice [1,3,4]. We have recently observed an acute effect of FGF19 on lowering blood glucose levels [10]. FGF21 was injected intraperitoneally (i.p.) at 5 mg/kg body weight into *ob*/*ob* mice and blood glucose levels were measured at 1, 3, 6, and 24 h post injection. Comparing to the PBS control group, similar to recent observation, a single i.p. injection of FGF21 resulted in a significant reduction in blood glucose levels, with approximately 40% reduction achieved 1 h post injection. This effect lasted up to 6 h and disappeared 24 h post injection (Fig. 1).

3.2. Acute FGF21 treatment inhibits lipolysis in primary adipocytes and differentiated 3T3L1 cells

We sought to explore potential mechanisms that might contribute to the acute lowering of plasma glucose levels by examining the effects of FGF21 on the functions of different cell types in vitro. We first tested effects of FGF21 on adipocyte functions given receptor expression and the well-established effects of FGF21 on the stimulation of glucose uptake into these cells [1]. The stimulated glucose uptake into 3T3L1 murine adipocyte cells reported previously was after 24 h treatment of the cells with FGF21 [1]. To explore whether this stimulation could occur acutely, we performed a time course experiment varying the length of FGF21 treatment prior to measuring the rate of glucose uptake. As shown in Fig. 2A, a treatment of 3T3L1 adipocytes with FGF21 for 4 h did not significantly stimulate glucose uptake into these cells. Significant stimulation was not observed until 72 h post FGF21 treatment (Fig. 2A). This is consistent with the previous hypothesis that transcriptional events, for example, the induction of GLUT1 expression, are needed to mediate the effects of FGF21 [1]. However, this suggests that the stimulation of glucose uptake into adipocyte may not fully explain the acute glucose lowering effect of FGF21 treatment in vivo.

Several previous reports also suggest that FGF21 may regulate adipocyte lipolysis as well [11,12] and adipocyte lipolysis could affect circulating FFA levels [13]. FFAs are not only energy substrates, but are also signaling molecules that may influence metabolic homeostasis. Elevated FFA levels could impair glucose and lipid metabolism in liver, muscle, pancreas, and might induce insulin

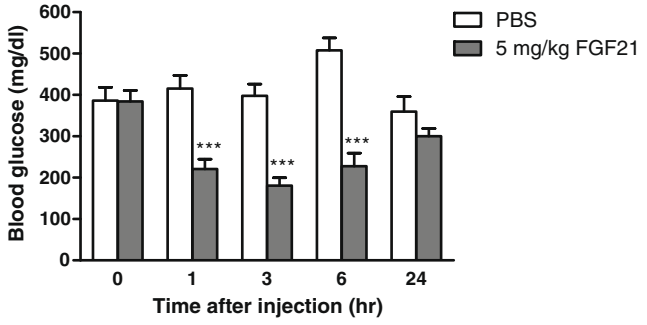

Fig. 1. Single injection of hFGF21 into *ob*/*ob* mice acutely reduces blood glucose. Age matched *ob*/*ob* mice were fed with normal chow overnight and their blood glucose measured in the morning. The mice were divided into two group (n = 10 in each group) based on their blood glucose and body weight. Compare to the control group (PBS at 5 ml/kg body weight), single injection of 1 mg/ml FGF21 (5 ml/kg body weight) acutely reduced the blood glucose. The glucose lower effect of FGF21 lasted more than 6 h in these mice. $^{***}P < 0.001$.

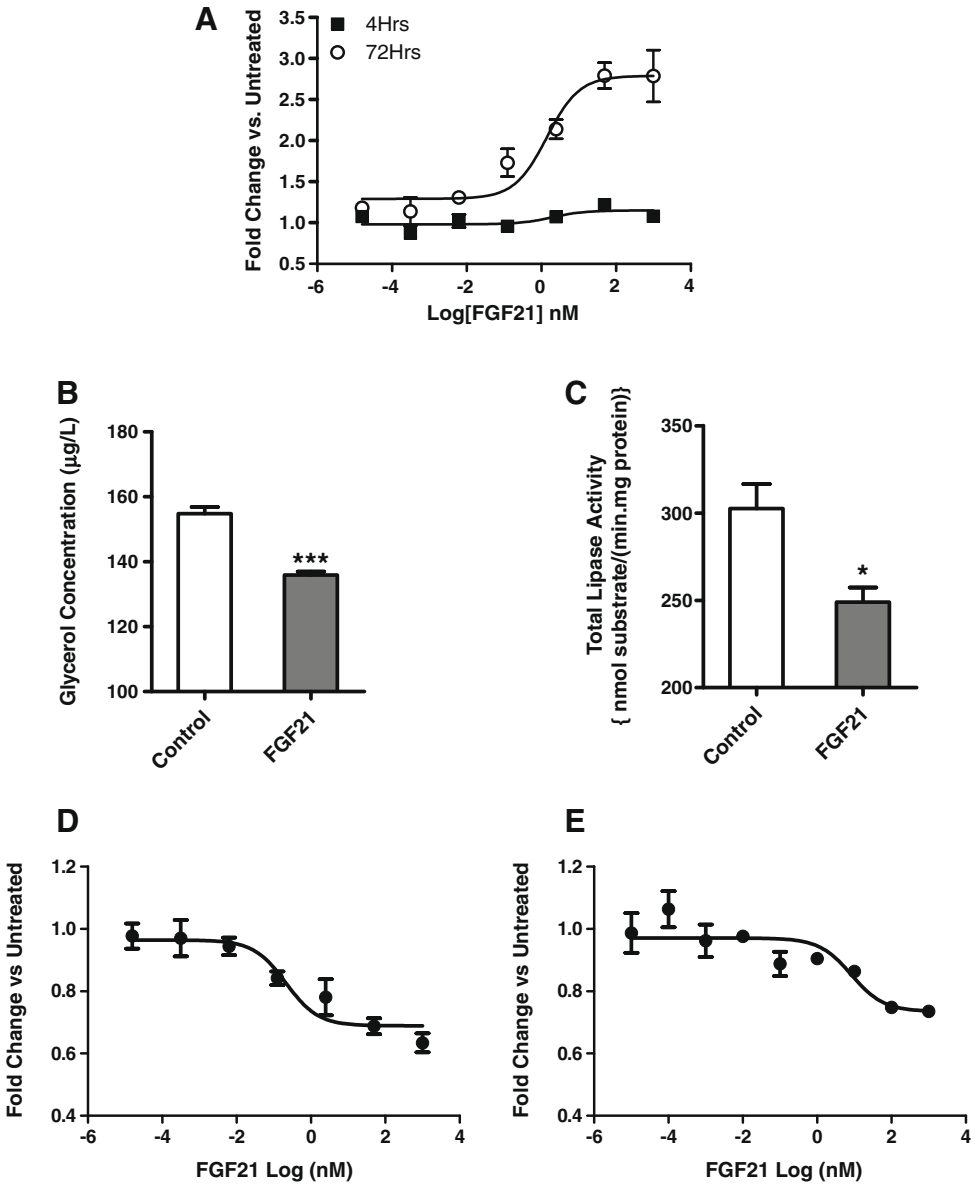

Fig. 2. Acute FGF21 treatment inhibits lipolysis in differentiated 3T3L1 cells and primary adipocytes. Differentiated 3T3L1 adipocytes (A, D, E) and isolated primary adipocytes (B and C) were treated with FGF21 or PBS. Glucose uptake in 3T3L1 cells was measured at 4 h or 72 h post FGF21 treatment (A). The acute effects of FGF21 treatments on lipolysis were measured 4 h post FGF21 treatment in primary adipocytes (B) and in 3T3L1 adipocytes (D). The lipolysis suppression effect is accompanied by the reduction of the totally lipase activity in primary adipocytes (C) and in 3T3L1 adipocytes (E). $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

resistance [14,15]. FFAs could also serve as liver substrate for gluconeogenesis and very low density lipoprotein production, and that in the diabetic state, gluconeogenesis is highly increased and contributes to the elevated blood glucose levels [16]. We, therefore, explored whether FGF21 could acutely regulate adipocyte lipolysis and whether this might contribute to the observed acute lowering of plasma glucose by FGF21 treatment in vivo.

Isolated mouse primary adipocyte cells were treated with FGF21 or PBS and the lipolysis rates in these cells were measured by the release of glycerol into the culture medium. As shown in Fig. 2B, FGF21 treatment inhibited lipolysis in mouse primary adipocytes. The inhibition of lipolysis by FGF21 could be observed as early as 1 h post treatment (data not shown) but was more pronounced between 4 and 6 h of treatment when 30% inhibition of lipolysis was observed (Fig. 2B). The lipolysis suppression effect is accompanied with the reduction of the total lipase activity in these cells (Fig. 2C). FGF21 treatment in differentiated 3T3L1 cells had the same inhibitory effects on lipolysis and total lipase activity (Fig. 2D and E). These data indicated that in adipose tissue, acute treatment of FGF21 leads to inhibition of total lipase activity and lipolysis.

3.3. Administration of FGF21 acutely lowered plasma FFA in both wild-type (wt) and ob/ob mice

Since the rate of adipocyte lipolysis could regulate plasma FFA levels, we next explored the acute effects of FGF21 treatment on plasma FFA levels in vivo. FGF21 was injected i.p. at 5 mg/kg body weight into both wt and *ob*/*ob* mice. Plasma samples were collected 0.5, 1 and 5 h after injection and FFA levels in the plasma were measured. Single injection of FGF21 into wt mice resulted in a rapid reduction in plasma FFA levels 30 min after the injection (Fig. 3A). The reduction in plasma FFA levels was also observed in *ob*/*ob* mice treated with FGF21, and the peak reduction was 1 h after injection, which corresponded to the timing of the glucose lowering effect of FGF21 (Fig. 3B). To provide further evidence

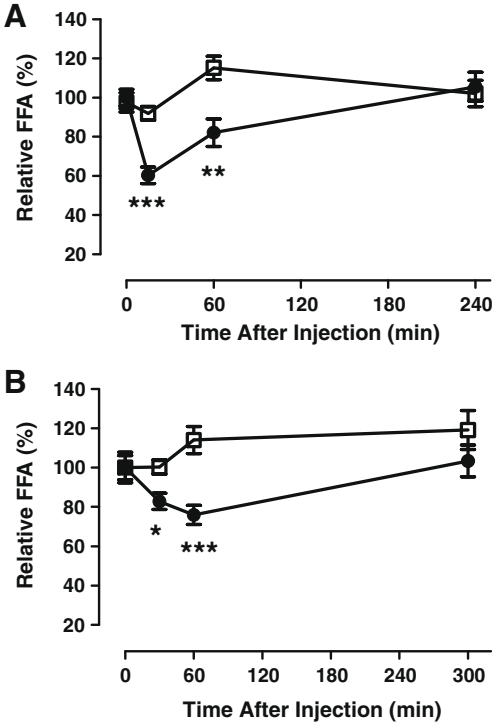

Fig. 3. Acute administration of FGF21 lowers plasma FFA in both wt and *ob*/*ob* mice. Age matched wt (A) or *ob*/*ob* (B) mice were fasted overnight. The mice were divided into two groups ($n = 10$ in each group) based on body weight. Compared to the control group (PBS at 5 ml/kg body weight), a single i.p. injection of 1 mg/ml FGF21 (5 ml/kg body weight) acutely reduced FFA levels in both wt (A) and *ob*/*ob* (B) mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

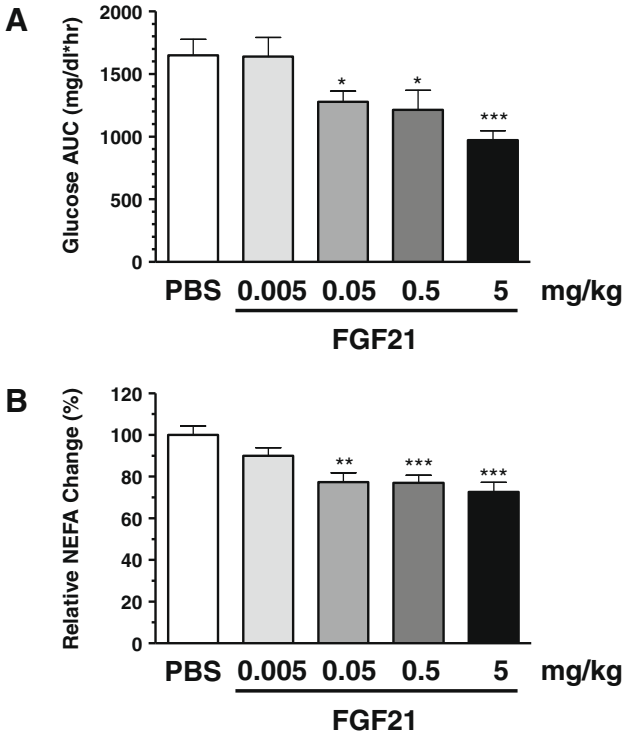

Fig. 4. Dose–response studies of FGF21 administration in *ob*/*ob* mice. Age matched *ob*/*ob* mice were divided into groups ($n = 10$ in each group) based on body weight. (A) Dose–response effects of FGF21 administration on plasma glucose levels. Mice were injected with indicated concentration of FGF21 (10 ml/kg body weight) similar to Fig. 1. Blood glucose levels were measured at 0, 1, 3, and 5 h post injection. The area under the curve (AUC) of the blood glucose during this period is shown. (B) Dose–response effects of FGF21 administration on plasma FFA levels. Mice were injected with indicated concentration of FGF21 (10 ml/kg body weight) similar to Fig. 3. Plasma FFA levels were measured before and 1 h after injection. The changes in FFA levels relative to PBS control group are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

for the potential link between the effects of FGF21 on plasma FFA and glucose, dose–response studies were carried out. FGF21 was injected i.p. between 0.005 mg/kg and 5 mg/kg body weight into *ob*/*ob* mice. As shown in Fig. 4, the potency of FGF21 induced reduction in plasma glucose levels (Fig. 4A) is consistent with the potency of its effects on reducing plasma FFA levels (Fig. 4B), and this is also in general agreement with previously published dose–responses of FGF21 in chronic studies [4,17]. This indicated that FGF21 treatment could suppress plasma FFA levels and this may have contributed to the acute reduction in blood glucose levels.

4. Discussion

FGF21 has emerged as an important metabolic regulator. Studies in multiple animal models have demonstrated the efficient and beneficial effects of FGF21 on glucose and lipid homeostasis. However, majority of the studies reported so far have been chronic studies, acute effects of FGF21 treatment have not been fully characterized. In this report, we studied the acute effects of FGF21 on plasma lipid parameters and found that a single FGF21 injection lowered plasma FFA levels similar to its acute effects on glucose as early as 1 h post injection in *ob*/*ob* mice.

In order to gain insights into the underlying mechanism for these observed acute effects, we explored the short term responses to FGF21 on metabolically relevant cell types. Since adipocytes have been shown to be a potential target tissue for FGF21, we explored whether regulation of adipocyte function might contribute to the observed acute effects of FGF21 in vivo. Consistent with previous reports, we show that significant glucose uptake into adipocyte does not occur post a transient treatment with FGF21. This agrees with the suggestion that stimulation of glucose uptake might be the result of transcriptional changes and upregulation of GLUT1 transporter in these cells [1]. This suggests that increased glucose uptake into adipocytes might not be the main contributor to the acute reduction in plasma glucose levels in vivo.

The reported effects of FGF21 on lipolysis has been inconsistent in the literature, both transient increase in lipolysis [11] and chronic inhibition of lipolysis [12] have been reported. To clarify the effects of FGF21 on adipocyte lipolysis, we examined the effects of FGF21 treatment on both the 3T3L1 adipocyte cells differentiated in vitro as well as primary adipocytes isolated from animals. Our results indicate that FGF21 treatment could inhibit adipocyte lipolysis after a short treatment in both the primary and the in vitro differentiated adipocyte cells. Such inhibition might be the result of inhibition of the lipase function as a reduction of total lipase activity was observed with FGF21 treatment (Fig. 2). Adipocyte lipolysis could regulate plasma FFA levels, and we have observed an acute reduction in plasma FFA levels post FGF21 injection in both wt and *ob*/*ob* mice (Fig. 3). The inhibition of adipocyte lipolysis by FGF21 in vitro could be observed as early as 1 h post treatment and become more pronounced at 4 h post treatment (Fig. 2). However, its ability to reduce plasma FFA levels peaked at 1 h post injection and disappeared at later time points (Fig. 3). This difference in timing of effects could be the result of differences in the state of adipocyte in vivo vs. in vitro. Alternatively, the stability of FGF21 in the different conditions could also contribute to the apparent disconnect in timing, the reported FGF21 plasma half life after a single i.v. or s.c. administration is less than 1 h in mice and its stability could be longer in culture media [5].

It has been reported that during the fasting state, more than 30% of gluconeogenesis comes from FFAs as substrates [13]. In diabetes, dysregulated insulin and glucagon levels and hepatic insulin resistance results in highly increased hepatic gluconeogenesis and contribute significantly to the elevated blood glucose levels [13]. Therefore, we propose that the acute reduction in plasma FFA levels post FGF21 treatment could lead to a reduction in gluconeogenesis. Lowered FFA levels may also improve muscle glucose utilization and liver insulin sensitivity [18]. This potential link is further supported by the similar dose–responses to FGF21 treatment in affecting plasma glucose and FFA levels (Fig. 4). The dose–responses of acute effects of FGF21 is also in general agreement with the dose–responses observed in chronic FGF21 studies reported previously on plasma glucose and lipid parameters [4,17], suggesting that the acute effects of FGF21 observed here may contribute to the chronic effects of FGF21. It is interesting to note that recent data may also support a direct effect of FGF21 on liver function [19]. Berglund et al. have recently reported the effects of acute 6 h infusion of FGF21 in *ob*/+ and *ob*/*ob* mice [19]. Their results showed a potent effect of FGF21 on the liver glucose flux and reported a reduction in liver triglyceride and an increase in liver glycogen content in the absence of changes in plasma insulin, FFA levels, or skeletal muscle and adipose glucose uptake, suggesting a direct effect of FGF21 on the liver [19]. It is possible that the combination of indirect effects on hepatic gluconeogenesis from the suppression of lipolysis and plasma FFA levels and a direct inhibition of glucose output from liver may explain the acute effect on lowering plasma glucose levels by FGF21.

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