Hepatic insulin resistance and increased hepatic glucose production in mice lacking *Fgf21*

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Abstract

Fibroblast growth factor 21 (FGF21) is an important regulator of hepatic glucose and lipid metabolism and represents a potential pharmacological agent for the treatment of type 2 diabetes and obesity. Mice fed a ketogenic diet (KD) develop hepatic insulin resistance in association with high levels of FGF21, suggesting a state of FGF21 resistance. To address the role of FGF21 in hepatic insulin resistance, we assessed insulin action in FGF21 whole-body knock-out (FGF21 KO) male mice and their littermate WT controls fed a KD. Here, we report that FGF21 KO mice have hepatic insulin resistance and increased hepatic glucose production associated with an increase in plasma glucagon levels. FGF21 KO mice are also hypometabolic and display increased fat mass compared with their WT littermates. Taken together, these findings support a major role of FGF21 in regulating energy expenditure and hepatic glucose and lipid metabolism, and its potential role as a candidate in the treatment of diseases associated with insulin resistance.

Key Words
- FGF21
- insulin resistance
- NAFLD
- energy expenditure
- obesity

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Introduction

The prevalence of obesity has dramatically risen over the last few decades and is not only affecting adults, but also children and adolescents (Finucane *et al.* 2011). Based on the National Health and Nutrition Examination Survey (NHANES), it is estimated that more than 34% of adults are obese and 68% are overweight or obese (Flegal *et al.* 2010). In parallel with obesity, nonalcoholic fatty liver disease (NAFLD), which is now the most prevalent chronic liver disease in the USA, is affecting about one out of three individuals (Williams *et al.* 2011). NAFLD is a major risk factor in the development of insulin resistance and type 2 diabetes (Angulo 2002, Fabbrini *et al.* 2010, Asrih & Jornayvaz 2015).

Fibroblast growth factor 21 (FGF21) is an important regulator of glucose metabolism (Kharitonenkov *et al.* 2005). FGF21 levels are increased in NAFLD and correlate with hepatic triglyceride content (Li *et al.* 2010); therefore, FGF21 is considered an emergent biomarker of NAFLD (Dushay *et al.* 2010, Morris-Stiff & Feldstein 2010). In diet-induced obese mice, which have already increased levels of FGF21, suggesting a state of FGF21 resistance, chronic administration of FGF21 reverses hepatic steatosis and improves insulin sensitivity (Xu *et al.* 2009, Camporez *et al.* 2013). Mice lacking *Fgf21* (FGF21 knock-out (KO)) gain weight in an age-dependent manner, with an increase in both fat and lean mass, and develop glucose intolerance

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on regular chow when assessed by intraperitoneal glucose tolerance tests (Badman *et al*. 2009). When fed a ketogenic diet (KD), FGF21 KO mice gain weight, develop NAFLD and impaired glucose control (Badman *et al*. 2009), although these findings have been questioned (Murata *et al*. 2013). Also, mice specifically lacking *Fgf21* in the liver fed a high-fat diet have recently been shown to develop significant hepatic steatosis and insulin resistance when assessed by an insulin tolerance test (Markan *et al*. 2014).

We previously showed that WT mice fed a KD develop hepatic insulin resistance despite gaining less weight than mice fed regular chow. In this case, hepatic insulin resistance was secondary to the accumulation of diacylgycerols (DAG), which are lipid intermediates known to activate protein kinase C epsilon (PKCε), which subsequently impairs insulin signaling (Samuel *et al*. 2007). Lower body weight in mice fed a KD was secondary to higher energy expenditure, which was attributed to an increase in FGF21 plasma concentrations, as caloric intake and locomotor activity were similar (Jornayvaz *et al*. 2010). We therefore hypothesized that FGF21 KO mice would have lower energy expenditure and develop more hepatic insulin resistance compared to WT mice when fed a KD. In order to examine this hypothesis, we assessed whole-body glucose turnover in awake mice using the hyperinsulinemic–euglycemic clamp technique combined with radiolabeled glucose, and energy expenditure by indirect calorimetry. In addition, we assessed liver lipid intermediates that have been associated with insulin resistance, such as triglycerides, DAG, and ceramides (Shulman 2000, Holland *et al*. 2007, Samuel *et al*. 2010, Jornayvaz & Shulman 2012), as well as signaling events typically associated with an increase in liver DAG content. Finally, given the well-established associations between endoplasmic reticulum (ER) stress and inflammation with insulin resistance (Ozcan *et al*. 2004, Hotamisligil 2006, 2010), we also assessed these pathways in FGF21 KO mice.

Materials and methods

Animals

Male FGF21 KO mice and WT littermates were generated as previously described (Potthoff *et al*. 2009) and individually housed under controlled temperature (23 °C) and lighting (12 h light:12 h darkness cycle, lights on at 0700 h) with free access to water and food. One week after arrival in the animal care facility, a KD (F3666, Bio-Serv, Frenchtown, NJ, USA) was started and continued for 5 weeks. The proportions of calories derived from nutrients were as follows: 95.1% fat, 0.4% carbohydrate, 4.5% protein, and energy density 7.456 kcal/g. Body composition was assessed by ^{1}H magnetic resonance spectroscopy using a Bruker Minispec Analyzer (Bruker BioSpin, Billerica, MA, USA). Energy expenditure, RQ, VO_2, VCO_2, locomotor activity, and food intake were measured using a Comprehensive Lab Animal Metabolic System (CLAMS; Colombus Instruments, Columbus, OH, USA). Drinking was measured as previously described (Birkenfeld *et al*. 2011). Mice were ~3 months old during the experiments. All experiments were done in 6-h fasted animals. All procedures were approved by the Yale University Animal Care and Use Committee.

Plasma assays

Blood samples were collected by cardiac puncture in heparinized syringes and centrifuged at 10,000 ***g*** for 2 min. Plasma was then either directly used or frozen at −20 °C for further analyzes. Plasma glucose (10 μl/sample) was measured using a YSI 2700D glucose analyzer (YSI, Inc., Yellow Springs, OH, USA). Plasma fatty acids were determined with the NEFA C Kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin, glucagon, and adiponectin were measured by RIA Kits (Millipore, Billerica, MA, USA). For glucagon measurements, aprotinin was added to the blood during collection to avoid degradation. Cholesterol panel was analyzed using COBAS Mira Plus (Roche). Plasma cytokines were measured using a mouse multiplex assay kit (Meso Scale Discovery, Gaithersburg, MD, USA).

Liver lipid intermediates measurements

Tissue triglycerides were extracted using the method of Bligh & Dyer (1959) and measured using a commercial triglyceride reagent (Diagnostic Chemicals Limited, Oxford, CT, USA). Cytosolic DAG were measured as previously described (Yu *et al*. 2002, Jornayvaz *et al*. 2011). Total DAG contents are expressed as the sum of individual species. Ceramides content was measured as previously described (Yu *et al*. 2002).

Hepatic glycogen measurements

Frozen livers were homogenized in perchloric acid. Glucose concentration was measured (YSI 2700D glucose analyzer; YSI, Inc.) in the raw homogenates and in hydrolyzed homogenates after the addition of

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amyloglucosidase from *Aspergillus niger* (Sigma–Aldrich). Results are expressed as mg of glycogen per 100 mg of tissue.

Hyperinsulinemic–euglycemic clamp studies

Jugular venous catheters were implanted 6–7 days prior to the hyperinsulinemic–euglycemic clamp experiments. Hyperinsulinemic–euglycemic clamps were conducted as previously described (Jornayvaz *et al*. 2011). Briefly, after overnight fasting, restrained mice were infused with [3-^{3}H]-glucose at a rate of 0.05 μCi/min for 120 min to measure basal glucose turnover. After the basal period, hyperinsulinemic–euglycemic clamp was performed for 120 min with a 4-min primed insulin (20 mU/kg) followed by a continuous infusion (3 mU/kg per min). [3-^{3}H]-glucose was infused at a rate of 0.1 μCi/min and 20% dextrose was infused in variable rates to maintain euglycemia (~120 mg/dl). 10 μCi bolus of 2-deoxy-D-[1-^{14}C]glucose (Perkin Elmer Life Sciences, Boston, MA, USA) was injected after 85 min to estimate the insulin-stimulated tissue glucose uptake. Blood samples were collected by tail bleeding (at 0, 25, 50, 65, 80, 90, 100, 110, and 120 min). Clamps were performed according to standard operating procedures (Ayala *et al*. 2010). At the end of the clamp, mice were anesthetized with pentobarbital sodium injection (150 mg/kg) and all tissues were taken within 4 min, snap–frozen in liquid nitrogen using aluminum tongs and stored at −80 °C for subsequent analysis. Biochemical analysis and calculations for the hyperinsulinemic–euglycemic clamps were performed as previously described (Jornayvaz *et al*. 2011).

Liver insulin signalling

PKCε membrane activation was assessed in liver protein extracts as previously described (Choi *et al*. 2007).

Immunoblot analysis

Immunoblots were done as previously described (Jornayvaz *et al*. 2012). Membranes were incubated overnight with primary antibodies for phospho-Akt2 (Ser^{474}) (Cell Signaling Technology, Danvers, MA, USA), phosphoenolpyruvate carboxykinase (PEPCK; Abcam, Cambride, MA, USA), pyruvate carboxylase (PC; Abcam), uncoupling protein 1 (UCP1; Santa Cruz Biotechnology), C/EBP homologous protein (CHOP; Cell Signaling Technology), IgH chain binding protein (BIP; Cell Signaling Technology), phospho-eIF2α (Cell Signaling Technology), or phospho-JNK (Cell Signaling Technology). After further washings, membranes were incubated with HRP-conjugated secondary antibody (Bio-Rad) and visualized by ECL substrate (Pierce, Rockford, IL, USA). Membranes were stripped and reblotted with anti-total Akt antibody (Cell Signaling Technology), total eIF2α (Cell Signaling Technology), total JNK (Cell Signaling Technology), or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology). Bands were then quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Total RNA preparation, real-time quantitative PCR analysis

Total RNA was extracted from frozen livers using RNeasy 96-Kit (Qiagen). Then, 1 μg of RNA was reverse-transcribed into cDNA with the use of the Quantitect RT Kit (Qiagen) as per manufacturer's protocol. The abundance of transcripts was assessed by real-time PCR on a 7500 Real-Time PCR System (Applied Biosystems) with a SYBR Green detection system. Samples were run in duplicate for both the gene of interest and cyclophilin, and data were normalized for the efficiency of amplification according to Pfaffl's equation (Pfaffl 2001), as determined by a standard curve included on each run. Primers used are available upon request.

Statistical analysis

Data are expressed as means ± S.E.M. Results were assessed using two-tailed unpaired Student's *t*-test or one-way ANOVA (GraphPad Prism 5, La Jolla, CA, USA). A P value <0.05 was considered significant.

Results

Decreased energy expenditure in FGF21 KO mice

Body weights of FGF21 KO mice studied at ~3 months of age were not significantly different than their WT littermate controls. Body fat expressed as a percentage of body weight was ~40% higher in FGF21 KO mice and there was no difference in their percent lean mass compared to controls (Table 1). However, when expressed in grams, lean mass was significantly lower in FGF21 KO mice (Table 1). VO_2 (Fig. 1A) and VCO_2 (Fig. 1B) were ~15% lower and energy expenditure was ~10% lower (Fig. 1C) in FGF21 KO mice than WT mice. Importantly, locomotor activity and caloric intake were similar between genotypes, as were respiratory quotient and drinking (Table 1). In accordance with the decrease in energy

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Table 1 Physiologic parameters and plasma analyses. Data are represented as mean±S.E.M.

	WT	FGF21 KO
Physiological parameters		
Body weight (g)	23.1±0.6	21.2±0.7
Fat mass (g)	2.0±0.1	2.6±0.3
Lean mass (g)	16.9±0.5	15.1±0.4*
Fat mass (% of body weight)	8.8±0.5	12.0±0.3*
Lean mass (% of body weight)	73.2±0.6	71.6±1.1
Caloric intake per mouse (kcal/(mouse-h))	0.57±0.06	0.49±0.06
RQ	0.73±0.00	0.73±0.00
Drinking (ml/(mouse-h))	0.01±0.00	0.01±0.00
Activity (counts/h)	65.6±10.8	80.9±13.8
Plasma analyses		
Fasting insulin (μU/ml)	9.9±1.7	15.4±4.6*
Clamp insulin (μU/ml)	61.3±12.2	73.1±8.3
Fasting glucagon (pg/ml)	118.7±38.6	245.1±23.0*
Fasting FA (mmol/l)	1.3±0.2	1.7±0.4*
Insulin-stimulated FA (mmol/l)	0.5±0.1	0.8±0.2*
Insulin suppression of FA (%)	63.0±5.5	54.4±6.8*
Total cholesterol (mg/dl)	181.6±20.2	291.7±20.3†
HDL cholesterol (mg/dl)	94.0±4.3	98.8±2.4
Triglycerides (mg/dl)	57.7±6.7	83.1±17.3
Adiponectin (μg/ml)	9.8±1.1	10.4±0.6
Interleukin 1 beta (pg/ml)	1.6±0.7	5.8±1.5‡
Interferon gamma (pg/ml)	1.0±0.2	1.1±0.1
Interleukin 6 (pg/ml)	6.6±0.7	8.2±2.3
Interleukin 10 (pg/ml)	43.2±1.6	50.3±3.4
Tumor necrosis factor alpha (pg/ml)	5.9±0.5	6.4±0.5

RQ, respiratory quotient; FA, fatty acids. $n=4$–12 mice/group. *$P<0.05$ and †$P<0.01$ vs WT. ‡$P=0.08$ vs WT.

expenditure, we found that protein levels of UCP1 in brown adipose, which is the main protein dissipating heat in the brown adipose tissue, was significantly reduced in FGF21 KO mice (Fig. 1D), corroborating the role of FGF21 in inducing UCP1 gene expression in brown adipose tissue (Fisher *et al.* 2012). Finally, basal plasma analyses revealed an ∼60% increase in total cholesterol in FGF21 KO, whereas there was no difference in HDL cholesterol and triglycerides (Table 1), suggesting a role for FGF21 in cholesterol metabolism.

Hepatic insulin resistance in FGF21 KO mice

We performed hyperinsulinemic–euglycemic clamps to assess hepatic and peripheral insulin sensitivity. Glucose infusion rates required to maintain euglycemia (Fig. 2A) during the clamps were ∼30% lower in FGF21 KO mice compared to WT mice, demonstrating whole-body insulin resistance in FGF21 KO mice (Fig. 2B). Basal endogenous glucose production was ∼20% higher in FGF21 KO mice (Fig. 2C) without any difference in basal plasma glucose, whereas the suppression of endogenous glucose production by insulin during the clamp was ∼60% lower in FGF21 KO mice (Fig. 2D), reflecting severe hepatic insulin resistance. However, insulin-stimulated whole-body glucose disposal (Fig. 2E) was similar between genotypes, demonstrating that the differences in whole-body glucose turnover were due specifically to hepatic insulin resistance in FGF21 KO mice. Basal plasma fatty acid concentrations were ∼30% higher in FGF21 KO mice and the ability of insulin to suppress plasma fatty acid concentrations during the hyperinsulinemic–euglycemic clamps was also significantly decreased in FGF21 KO mice (Table 1), suggesting that insulin suppression of white adipose tissue lipolysis was also impaired in these mice. Fasting plasma insulin concentrations were ∼55% higher in FGF21 KO mice, whereas insulin concentrations during

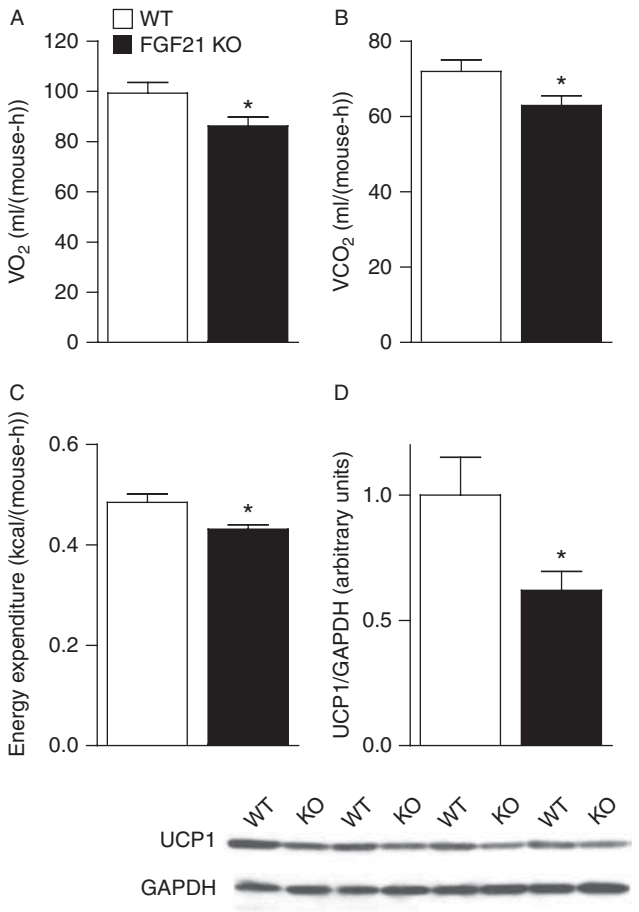

Figure 1
Decreased energy expenditure in FGF21 KO mice. VO_2 (A), VCO_2 (B), and energy expenditure per mouse (C) were significantly lower in FGF21 KO mice ($n=10$–12/group). Accordingly, the protein level of UCP1 (D) was significantly decreased in FGF21 KO mice ($n=4$–5/group). *$P<0.05$ vs WT mice. Data are represented as mean±S.E.M.

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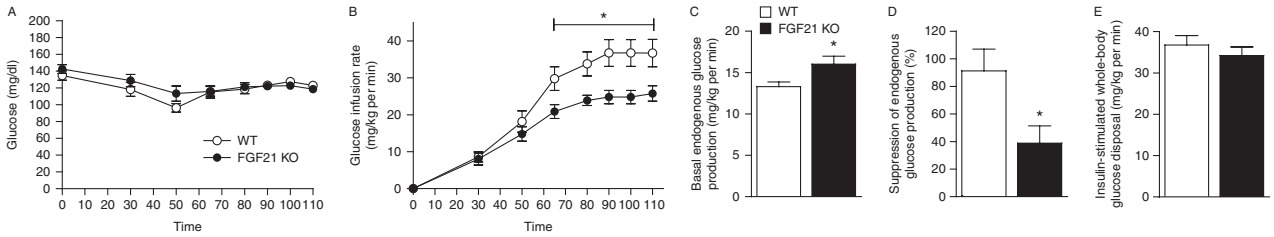

Figure 2
Hepatic insulin resistance in FGF21 KO mice. (A) Glucose was maintained around 100–120 mg/dl during the hyperinsulinemic–euglycemic clamps. Glucose infusion rates (B) were significantly lower in FGF21 KO mice compared to their WT littermate controls. Basal endogenous glucose production (C) was higher in FGF21 KO mice. FGF21 KO mice displayed hepatic insulin resistance as reflected by the inability of insulin to suppress endogenous glucose production during the hyperinsulinemic–euglycemic clamps (D). Insulin-stimulated whole-body glucose disposal (E) was similar between groups ($n=9$–10/group). *$P<0.05$ vs WT mice. Data are represented as mean ± S.E.M.

the clamp were similar between genotypes (Table 1), which is important to allow clamp data comparisons between groups.

To assess the role of FGF21 deficiency in insulin resistance *in vitro*, we measured FGF21 expression in HepG2 cells in basal conditions and found that it was very low (data not shown), suggesting that knocking down FGF21 would have little effect in these cells. These findings are in line with previous studies in different hepatic cell lines (Potthoff *et al*. 2009, Wang *et al*. 2010, Schaap *et al*. 2013) and overall suggest that the role of FGF21 in insulin resistance is more relevant in *in vivo* systems.

NAFLD and impaired hepatic insulin signaling in FGF21 KO mice

FGF21 KO mice, like WT mice, develop NAFLD when fed a KD. However, liver lipid intermediates, triglycerides (Fig. 3A), cytosolic DAG (Fig. 3B), and ceramides (Fig. 3C) were all significantly higher in FGF21 KO mice. The increase in hepatic DAG content was associated with a significant increase in PKCε membrane translocation (Fig. 3D) and a subsequent ∼10% decrease in phospho-Akt2 phosphorylation (Fig. 3E), confirming hepatic insulin resistance in FGF21 KO mice downstream of the insulin receptor.

The interaction of FGF21 with β-klotho is necessary for its biological action (Kharitonenkov & Larsen 2011, Ding *et al*. 2012). We therefore assessed part of FGF21 signaling in the liver. Interestingly, although mice fed a KD are known to display altered FGF21 signaling compared to mice fed regular chow (Asrih *et al*. 2015), we found no difference in β-klotho (Supplementary Figure 1A, see section on supplementary data given at the end of this article), FGF receptor 1 (Supplementary Figure 1B), and 4 (Supplementary Figure 1C) hepatic mRNA expression.

ER stress and inflammation in FGF21 KO mice

ER stress and inflammation are also considered as alternative hypotheses to explain hepatic insulin resistance (Ozcan *et al*. 2004, Hotamisligil 2006, Asrih & Jornayvaz 2013). We therefore examined several markers of these pathways. Hepatic protein levels of CHOP (Fig. 4A), BIP (Fig. 4B), two important markers of ER stress, were similar between groups. However, phospho-eIF2α, another ER stress marker, was increased by ∼35% in FGF21 KO mice (Fig. 4C). Protein levels of phospho-JNK, an important marker of inflammation, were similar between groups (Fig. 4D). Additionally, concentrations of plasma cytokines, namely interleukin 6 (IL6), interferon gamma, IL10, and tumor necrosis factor alpha were not different between FGF21 KO and WT mice (Table 1). However, IL1β tended to be higher in FGF21 KO mice ($P=0.08$; Table 1). Finally, adiponectin, which has been suggested to activate ceramide catabolism (Holland *et al*. 2011), as well as mediate part of FGF21 metabolic effects (Holland *et al*. 2013, Lin *et al*. 2013), although this has been recently challenged (Muise *et al*. 2013, Markan *et al*. 2014), was similar between groups (Table 1).

Increased hepatic gluconeogenesis in FGF21 KO mice

In order to understand why FGF21 KO mice had a higher basal endogenous glucose production than WT mice, we assessed different enzymes involved in gluconeogenesis. Of note, *Fgf21* mRNA expression was decreased in FGF21 KO mice, according to their genotype (Fig. 5A). The expression of peroxisome proliferator activated receptor gamma coactivator 1 alpha (*Pgc1α*) was not significantly

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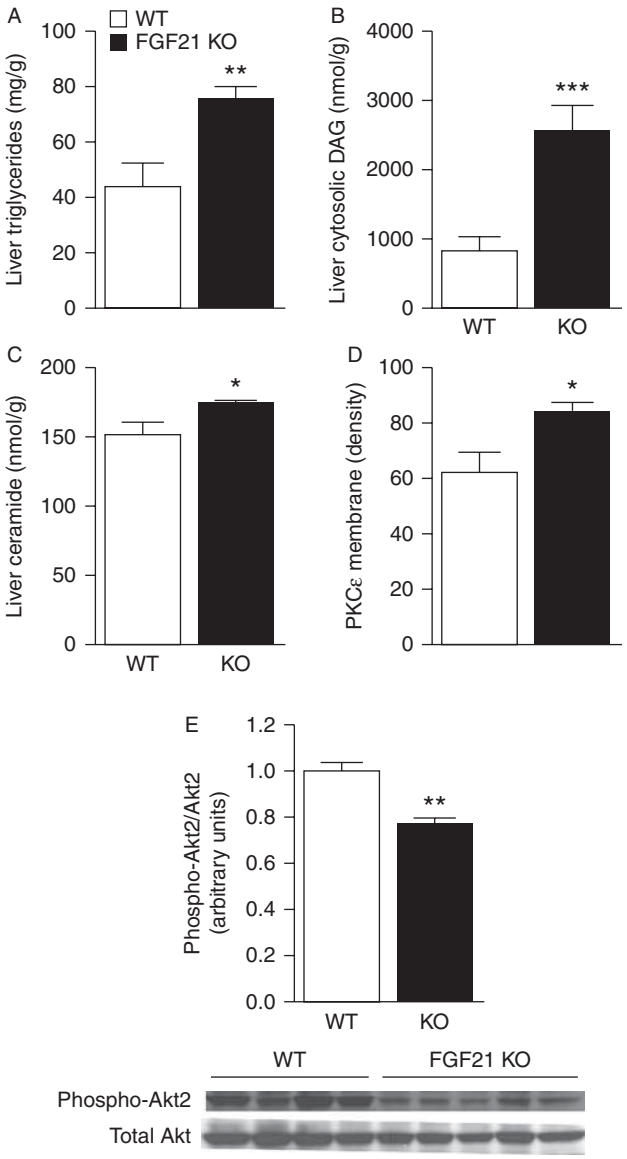

Figure 3
NAFLD and impaired hepatic insulin signaling in FGF21 KO mice. (A, B and C) Liver triglycerides (A), cytosolic DAG content (B), and ceramides content (C) were significantly increased in FGF21 KO mice ($n=8$–10/group). Consequently, PKCε was significantly translocated to the membrane (D) in FGF21 KO mice and insulin-stimulated Akt2 phosphorylation was decreased (E) ($n=4$–5/group). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs WT mice. Data are represented as mean ± S.E.M.

altered, nor were mRNA expression of *Pepck*, *Pc*, and glucose-6-phosphatase (*G6pase*) (Fig. 5A). However, at the protein level, we found a significant increase in the expression of *PEPCK* (Fig. 5B), but not *PC* (Fig. 5C), in FGF21 KO mice. In association with these findings and the increased hepatic glucose production in FGF21 KO mice, plasma glucagon concentrations were more than twofold higher in FGF21 KO mice (Table 1). Finally, we found a decrease in hepatic glycogen content in FGF21 KO mice (Fig. 5D), suggesting a decrease in glycogen synthesis or an increase in glycogenolysis.

Discussion

FGF21 is rapidly gaining attention as a potent metabolic regulator that affects both glucose and lipid metabolism and reduces body weight and fat mass in numerous animal models of insulin resistance and obesity (Kharitonenkov *et al*. 2005, 2007, Coskun *et al*. 2008, Berglund *et al*. 2009, Xu *et al*. 2009, Camporez *et al*. 2013). FGF21 plasma concentrations are also correlated with insulin resistance in humans when assessed by the hyperinsulinemic–euglycemic clamp technique (Chavez *et al*. 2009), which represents the gold standard to evaluate insulin sensitivity. FGF21 also emerges as a biomarker of NAFLD (Li *et al*. 2010, Morris-Stiff & Feldstein 2010), a highly prevalent disease in western countries. These findings of increased FGF21 levels in humans with insulin resistance actually suggest a state of FGF21 resistance. Nevertheless, these findings also suggest that giving supra-physiological doses of FGF21 could be used for the treatment of insulin resistance and other aspects of the metabolic syndrome in humans (Kliewer & Mangelsdorf 2010).

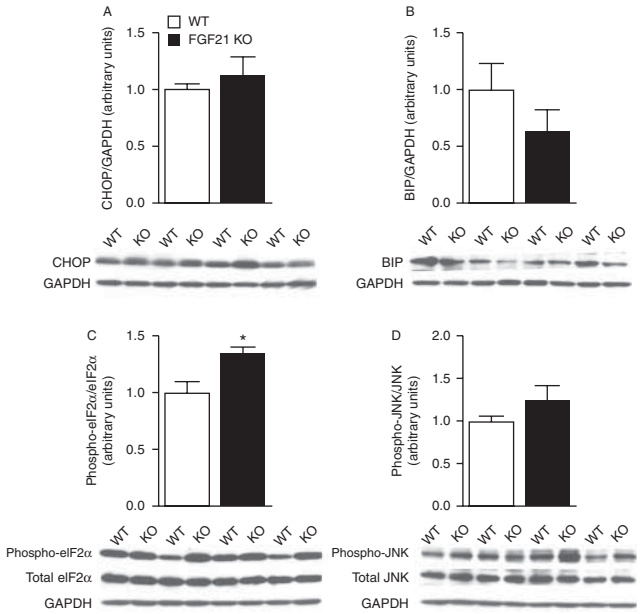

Figure 4
ER stress and inflammation in FGF21 KO mice. Major markers of ER stress, CHOP (A) and BIP (B) were not different between FGF21 KO and WT mice, whereas phospho-eIF2α (C) was significantly higher in FGF21 KO mice. Phospho-JNK (D) was not different between genotypes ($n=4$–5/group). $^{*}P<0.05$ vs WT mice. Data are represented as mean ± S.E.M.

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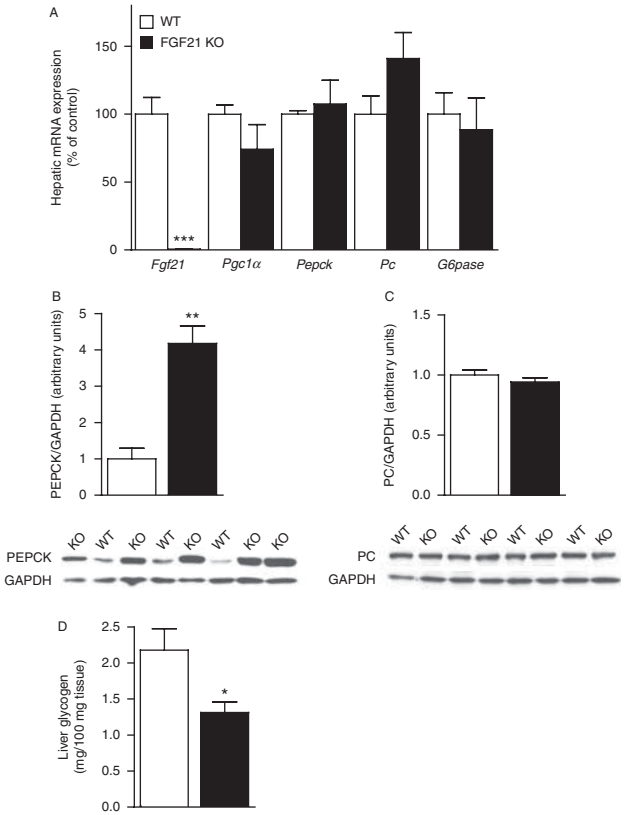

Figure 5

Increased hepatic gluconeogenesis in FGF21 KO mice. According to their genotypes, mRNA level of *Fgf21* (A) were significantly decreased in FGF21 KO mice. There was no difference in the mRNA expression of *Pgc1α*, *Pepck*, *Pc*, and *G6pase* between FGF21 KO and WT mice (A) ($n=6$/group). However, protein levels of PEPCK (B) were significantly increased in FGF21 KO mice, whereas there was no difference in PC protein levels (C) ($n=4$–5/group). Finally, hepatic glycogen content (D) was significantly lower in FGF21 KO mice ($n=8$–9/group). *$P<0.05$ and **$P<0.01$ vs WT mice. Data are represented as mean ± S.E.M.

Notably, a promising study in obese type 2 diabetic patients using FGF21 analogs during 28 days reported a trend in decreased plasma glucose levels and changes in plasma insulin levels, suggesting improved insulin sensitivity (Gaich *et al.* 2013).

Here, we report that mice lacking *Fgf21* have severe hepatic insulin resistance when fed a KD compared with WT controls, when assessed by the gold-standard technique, the hyperinsulinemic–euglycemic clamp. Our results are consistent with a previous report of glucose intolerance in FGF21 KO mice, although the intraperitoneal glucose tolerance tests were not done in body weight-matched animals (Badman *et al.* 2009), which does not represent ideal conditions (Ayala *et al.* 2010) and does not distinguish hepatic vs peripheral insulin sensitivity directly. Our results are also in line with a recent study showing impaired insulin sensitivity assessed by insulin tolerance tests in mice specifically lacking *Fgf21* in the liver (Markan *et al.* 2014). However, another group recently reported opposite findings, i.e., improved glucose tolerance using intraperitoneal glucose tolerance tests in FGF21 KO mice (Murata *et al.* 2013). The reasons for this are unclear, but could be due to a different line of mice, the different KD used, as well as the different duration of KD feeding, which was only 6 days in the study by Murata *et al.* (2013), therefore contrasting with previous studies (Badman *et al.* 2009, Jornayvaz *et al.* 2010), including ours. Moreover, their mice did not develop fatty liver on a KD, as opposed to our results and those of others (Badman *et al.* 2009, Markan *et al.* 2014).

Insulin resistance in our FGF21 KO mice was associated with an almost threefold increase in hepatic cytosolic DAG content leading to PKCε activation, resulting in decreased insulin signaling as shown by decreased Akt2 phosphorylation, confirming the known impairment of insulin signaling by PKCε activation (Jornayvaz & Shulman 2012). Hepatic ceramide content was also slightly increased (~15%) which may have also contributed to the hepatic insulin resistance observed in these mice. Also, increased basal plasma concentrations of fatty acids and impaired suppression by insulin might have played a role in the development of insulin resistance in FGF21 KO mice. This suggests that FGF21 is required to inhibit lipolysis in white adipose tissue and is consistent with previous findings (Arner *et al.* 2008, Hotta *et al.* 2009, Li *et al.* 2009, Murata *et al.* 2013). For these experiments, we decided to feed mice a KD because we previously reported that such a diet induces hepatic insulin resistance in WT mice with high plasma levels of FGF21 (Jornayvaz *et al.* 2010), consistent with the elevation of FGF21 seen in insulin resistance in humans (Chavez *et al.* 2009) and mice (Fisher *et al.* 2010, Jornayvaz *et al.* 2010). As WT mice are insulin resistant when fed a KD, we now show that mice lacking *Fgf21* are even more insulin resistant when fed the same diet. However, these studies raise the question as to whether the increase in FGF21 on a KD contributes to the development of insulin resistance or if it is a counter regulatory mechanism to promote the use of lipids during this challenge. Our experiments test this directly and show that FGF21 provides some level of protection against insulin resistance. However, the increase in FGF21, whether mice are fed a KD (Jornayvaz *et al.* 2010) or a high-fat diet (Fisher *et al.* 2010), is insufficient to protect against insulin resistance. However, KD has been shown to increase FGF21 levels because FGF21 is an endocrine signal of protein restriction (Laeger *et al.* 2014). KD can therefore

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increase FGF21 independently of insulin resistance. The increase in hepatic lipid content in FGF21 KO mice is consistent with a role of FGF21 in the regulation of hepatic lipid metabolism (Badman *et al.* 2007). Specifically, mice lacking *Fgf21* fed a KD are known to have an increase in hepatic protein levels of sterol regulatory binding protein element 1c (*SREBP1c*; Badman *et al.* 2009), a master regulator of lipid synthesis.

Interestingly, when fed a KD, FGF21 KO mice had a decrease in energy expenditure, and this is a likely explanation of why their body weight increases with age. These results confirm data from previous reports where FGF21 was shown to act as a regulator of energy metabolism (Coskun *et al.* 2008, Xu *et al.* 2009, Camporez *et al.* 2013). The effect of FGF21 on energy expenditure is likely to be direct, as we found no difference in locomotor activity, food consumption, or respiratory quotient. Moreover, we found that the level of UCP1, the main protein dissipating heat at the level of the brown adipose tissue, was significantly reduced in FGF21 KO mice. However, by surgically removing brown adipose tissue, we previously showed that FGF21 can also increase energy expenditure independently of brown adipose tissue, notably in white adipose tissue, suggesting that FGF21 can increase energy expenditure in other organs (Camporez *et al.* 2013).

We also found evidence of a potential role of FGF21 on ER stress as evidenced by an increase in phospho-eIF2α. Phospho-eIF2α, an important marker of ER stress, was significantly increased in FGF21 KO mice. It is not clear why this marker of ER stress of the PERK arm was increased, whereas CHOP, another ER stress marker from the PERK arm, was not. However, this elevation may be secondary to higher fat content in the liver of FGF21 KO mice compared to WT mice. Moreover, this corroborates findings in obese non-diabetic patients with NAFLD (Kumashiro *et al.* 2011). Finally, adiponectin, which is thought to mediate insulin sensitivity by activating ceramidase activity (Holland *et al.* 2011) and has recently been shown to mediate part of FGF21 metabolic actions (Holland *et al.* 2013, Lin *et al.* 2013), was not different between groups and was therefore unlikely to play a major role in the hepatic insulin resistance seen in mice lacking *Fgf21*. These findings are therefore in line with recent studies suggesting that other factors than adiponectin mediate FGF21 effects (Muise *et al.* 2013, Markan *et al.* 2014).

An important finding here was that mice lacking *Fgf21* had an increase in endogenous glucose production, which can be the result of both increased gluconeogenesis and/or glycogenolysis. We found no difference in the mRNA expression of major gluconeogenic enzymes, but found a significant increase in *PEPCK* protein level, providing evidence for increased gluconeogenesis. It is not surprising that mRNA levels were not altered, as it has been shown that increased transcriptional expression of *Pepck* does not account for increased gluconeogenesis and fasting hyperglycemia in patients with type 2 diabetes. Indeed, hepatic expression of *Pepck* was not different between insulin sensitive and type 2 diabetic subjects (Samuel *et al.* 2009). Interestingly, a study reported *in vitro* in Hepa1–6 hepatocytes that downregulation of *Fgf21* increases *Pepck* expression, whereas upregulation of *Fgf21* decreases *Pepck* (Li *et al.* 2012). The latter finding was further confirmed in the rat H4IIE hepatoma cell line (Kong *et al.* 2013). Therefore, other mechanisms associated with lack of *Fgf21* that have yet to be unraveled potentially affect the post-transcriptional modification of PEPCK, which suggests that FGF21 plays an important role in gluconeogenesis. We also report decreased hepatic glycogen content in FGF21 KO mice, which could reflect decreased glycogen synthesis or increased glycogenolysis. The latter would be more likely, based on the increased endogenous glucose production evidenced in FGF21 KO mice, and is further corroborated by the higher plasma glucagon levels in these mice, although these findings remain associative. This increase in plasma glucagon levels contrasts with previous reports (Badman *et al.* 2009, Potthoff *et al.* 2009, Murata *et al.* 2013), but might reflect the different diet used and the duration of the diet. Also, glucagon is rapidly destroyed in plasma; we therefore used aprotinin during blood collection to avoid degradation of the hormone. However, our results mirror findings in mice overexpressing *Fgf21*, where plasma glucagon levels were significantly lower in females and tended to be lower in males (Kharitonenkov *et al.* 2005). This increase in glucagon might be a compensatory mechanism to increase FGF21 concentrations, as recent findings reported that glucagon increases FGF21 levels (Arafat *et al.* 2013), further mediating glucagon actions (Habegger *et al.* 2013), but these considerations remain speculative, as we have no direct evidence. Nevertheless, glucagon levels have been reported to be lowered after FGF21 injections in *ob/+* (Berglund *et al.* 2009) and *db/db* mice (Mu *et al.* 2012), as well as in diabetic monkeys (Kharitonenkov *et al.* 2007) and in isolated rat pancreatic islets (Kharitonenkov *et al.* 2005). Finally, the increased basal plasma insulin concentrations in FGF21 KO mice may be secondary to the increased endogenous glucose production.

In conclusion, the present study demonstrates that mice specifically lacking *Fgf21* develop hepatic insulin

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resistance when fed a KD, thereby involving FGF21 as a regulator of hepatic insulin resistance. In this case, hepatic insulin resistance can be attributed to an increase in hepatic DAG content, leading to PKCε activation and subsequent impaired insulin signaling. Taken together, these findings support the hypothesis that FGF21 plays a key role in NAFLD-associated hepatic insulin resistance and provide new support of FGF21 as a pharmacological candidate in insulin resistance and associated diseases.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0136.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J P G C designed experiments, researched data, and wrote manuscript. M A researched data, contributed to discussion, and reviewed/edited manuscript. D Z researched data. M K researched data. V T S contributed to discussion and reviewed/edited manuscript. M J J designed experiments, researched data, contributed to discussion, and reviewed/edited manuscript. F R J designed experiments, researched data, reviewed/edited manuscript, contributed to discussion, and wrote manuscript. All authors approved the final version. F R J is the guarantor of this work.

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