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## **Dual effects of Fibroblast Growth Factor 21 on hepatic energy metabolism**

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## **Abstract**

The aim of this study was to investigate the mechanisms by which FGF21 affects hepatic integration of carbohydrate and fat metabolism in Siberian hamsters, a natural model of adiposity. Twelve aged matched adult male Siberian hamsters maintained in their long day fat state since birth were randomly assigned to one of two treatment groups and were continuously infused with either vehicle (saline; n=6) or recombinant human FGF21 protein (1 mg/kg/day; n=6) for 14 days. FGF21 administration caused a 40% suppression ( $P<0.05$ ) of hepatic pyruvate dehydrogenase complex (PDC), the rate-limiting step in glucose oxidation, a 34% decrease ( $P<0.05$ ) in hepatic acetylcarnitine accumulation, an index of reduced PDC flux, a 35% increase ( $P<0.05$ ) in long-chain acylcarnitine content (an index of flux through  $\beta$ -oxidation) and a 47% reduction ( $P<0.05$ ) in hepatic lipid content. These effects were underpinned by increased protein abundance of PD kinase-4 (a negative regulator of PDC), the phosphorylated (inhibited) form of acetyl-CoA carboxylase (ACC, a negative regulator of delivery of fatty acids into the mitochondria), and the transcriptional co-regulators of energy metabolism peroxisome proliferator activated receptor gamma co-activator alpha (PGC1 $\alpha$ ) and sirtuin-1 (SIRT-1). These findings provide novel mechanistic basis to support the notion that FGF21 exerts profound metabolic benefits in the liver by modulating nutrient flux through both carbohydrate (mediated by a PDK4-mediated suppression of PDC activity) and fat (mediated by deactivation of ACC) metabolism, and therefore may be an attractive target for protection from increased hepatic lipid content and insulin resistance that frequently accompany obesity and diabetes.

## **Introduction**

Fibroblast growth factor 21 (FGF21) is an endocrine member of the fibroblast growth factor superfamily that plays a key role in the regulation of carbohydrate and fatty acid metabolism (Kharitonov and Adams 2014). The primary physiological role of FGF21 is reported to occur during the adaptive response

to starvation in rodents (Markan, et al. 2014). Hepatic derived FGF21 has been shown to function in an endocrine manner to facilitate hepatic fatty acid mitochondrial delivery,  $\beta$ -oxidation and subsequently ketone body production (Badman, et al. 2007; Markan et al. 2014; Potthoff, et al. 2009). In accordance with this, the administration of recombinant human FGF21 to animal models of obesity and diabetes was shown to confer clear metabolic benefits, including lowering of plasma glucose and insulin concentrations, improved blood lipid profiles and significant body weight reduction (Adams, et al. 2012; Coskun, et al. 2008; Kharitononkov, et al. 2005). Consequently FGF21 is now being evaluated as a potential treatment for both obesity and diabetes and their associated comorbidities (Gaich, et al. 2013; Gimeno and Moller 2014).

The Siberian hamster (*Phodopus sungorus*) is a natural animal model of adaptive adiposity, which can be experimentally induced via a change in photoperiod to increase or decrease appetite and body weight (Ebling 2014). Their summer state is induced by long day lengths and is characterised by increased adiposity coupled with leptin resistance and elevated plasma insulin concentrations. In the winter short day lean state, the animals exhibit reduced plasma leptin concentrations and whole-body fat mass (specifically abdominal white adipose tissue). Furthermore, it has been demonstrated that these changes in the winter state are due to reduced daily food intake and increased whole-body daily energy expenditure (Warner, et al. 2010). We recently reported that plasma levels of FGF21, along with its local FGF21 protein content in liver and brown adipose tissue, increase in the Siberian hamster when transitioning from its long day fat state to that of its short day lean state, suggesting that FGF21 may play a role in facilitating the shift in metabolic phenotype (Samms, et al. 2014). In support of this, our studies demonstrated that treatment of the Siberian hamster with recombinant FGF21 reduced body weight due to an increase in whole-body energy expenditure, which was associated with a switch in metabolic fuel utilisation from carbohydrate to fat oxidation (Murphy, et al. 2013).

In addition to its whole body effects, FGF21 has also been shown to have distinct local effects in the liver. Both whole-body and liver specific deletion of FGF21 in mice leads to increased hepatic triglyceride content (Markan et al. 2014). Conversely, exogenously administered FGF21 was reported to lower hepatic fat content and improve hepatic insulin sensitivity when administered to animal models suffering with fatty liver disease (Berglund, et al. 2009; Camporez, et al. 2013). At the molecular level these effects appear to be associated with the up-regulation of transcriptional regulators of hepatic fatty acid oxidation and down-regulation of regulators of hepatic fatty acid synthesis (Adams et al. 2012; Coskun et al. 2008). Although these data provide some molecular insight, the exact mechanisms by which FGF21 induces its effects on hepatic metabolism (and in particular its effects on carbohydrate metabolism) and consequently how this then results in protection from increased hepatic lipid content is not well understood. Liver is one of the primary targets of FGF21 under both physiological (Adams, et al. 2010; Badman et al. 2007) and pharmacological conditions (Berglund et al. 2009; Fisher, et al. 2011) and, as indicated by our recent findings, appears to be a primary source of systemic endogenous FGF21 availability under physiological conditions (Samms et al. 2014). Given the crucial role of liver in the regulation of energy metabolism and insulin action, FGF21 be an attractive target for protection from increased hepatic lipid content and insulin resistance that frequently accompany obesity and diabetes. Thus, the aim of the present study was to investigate the biochemical and molecular pathways responsible for the FGF21-mediated decrease in hepatic triglyceride content focusing on regulation of the integration of hepatic carbohydrate and fatty acid metabolism. We used Siberian hamsters maintained in their long-day high body weight state since our recent studies have reported that the metabolic effects of recombinant FGF21 are far more efficacious in this species when maintained in its fat state compared to its lean state (Murphy et al. 2013). The chronic infusion paradigm was designed to mimic the natural increase in circulating FGF21 that occurs when animals are exposed to SD (Samms et

al. 2014). The data from this study further emphasise the potential of FGF21 as a therapeutic for the treatment of obesity and diabetes.

## **Materials and methods**

**Animal housing.** Adult male animals were obtained from a colony of Siberian hamsters (*Phodopus sungorus*) maintained at the University of Nottingham Biomedical Services Unit (Ebling 1994). All studies were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 (project licence: PPL 40/3604) and approved by the University of Nottingham Animal Welfare and Ethical Review Board. Hamsters were group housed at approximately 21°C and 40% humidity, and were allowed *ad libitum* access to water and standard laboratory chow comprising of 19% protein, 45% carbohydrate, 9% fat (Teklad 2019, Harlan, UK). Animals were housed from birth in long day conditions (LD) of 16 hours light: 8 hours dark with lights off at 11:00 GMT.

**Experimental design.** The effects of continuously infused recombinant human FGF21 protein (Kharitononkov et al. 2005) were evaluated in aged matched (~ 6 months) adult male Siberian hamsters that had been housed in LD since birth (n=12). Sample size was determined by power calculation on the basis of our previous experiments using the reduction in body weight as the primary experimental endpoint. Body weight and food intake were recorded on a weekly basis prior to the start of the experimental period. Animals were then randomly assigned (based on initial body mass using an online randomization plan generator software) to one of two treatment groups (with no blinding performed) in which Alzet osmotic mini-pumps (model 2002, Charles River) were subcutaneously implanted and set to release vehicle (saline; n=6) or FGF21 (1 mg/kg/day; n=6) for 14 days. The osmotic minipumps were inserted below the skin on the flank of the hamster under isoflurane anaesthesia. Hamsters were treated with an analgesic (5 mg/kg s.c., Rimadyl, Pfizer Animal Health, Kent, UK), and the wound closed with Michel clips. Post-surgery, hamsters were treated daily for 3 days with an analgesic at 5 mg/kg body

weight (Rimadyl, as above) and additional fluids consisting of 500  $\mu$ l/day 0.9% sterile saline (Baxter Healthcare, Thetford, UK), in accordance with LASA (Laboratory Animal Science Association) guidelines. Body weight and food intake were recorded on a daily basis. Seven days after surgery animals were transferred to metabolic cages (CLAMS) for a 48 hour period with the first 24 hours used as a habituation period and the final 24 hours for the measurement of the metabolic parameters described in the section below. Fourteen days after the start of the experimental period hamsters were euthanized by intraperitoneal injection of sodium pentobarbitone (Euthatal: Rhone Merieux, Harlow, UK) and liver tissues were collected, immediately frozen in liquid nitrogen and subsequently stored at  $-80^{\circ}\text{C}$  until the biochemical and molecular analyses described below were conducted.

**Whole-body metabolic analysis.** Oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) were measured concurrently using a modified open circuit calorimeter known as the CLAMS, as described previously (Warner et al. 2010).  $\text{VO}_2$  and  $\text{VCO}_2$  were then used to calculate energy expenditure and the rate of whole body carbohydrate (COX) and fat (FOX) oxidation, as previously described (Frayn 1983). Measurements were taken at 9-min intervals for 24 h, commencing 7 days after the start of FGF21 treatment. Hamsters had *ad libitum* access to water and ground chow when in the CLAMS. All measurements were made at room temperature which was maintained at approximately  $21^{\circ}\text{C}$ .

**Liver metabolites.** Two hundred to 250 mg of frozen 'wet' liver tissue was freeze-dried and powdered. One part of the freeze-dried tissue (20-30 mg) was used for the determination of acetylcarnitine and long-chain acylcarnitine content using radioenzymatic methods previously described (Cederblad, et al. 1990). An aliquot of the freeze-dried liver tissue (~20-30 mg) was also used for the quantitative determination of triglyceride (TG) content. Briefly, the tissue total lipid content was extracted using the method by Folch et al (Folch, et al. 1957), followed by removal of phospholipids by adding 250 mg of silicic acid, and saponification of TGs to glycerol and FFAs using a mixture of KOH and ethanol (5:95).

Samples were then centrifuged and the resulting supernatants used for the enzymatic determination of glycerol. Approximately 10-15 mg of the 'wet' liver was also used to determine hepatic dehydrogenase complex activity expressed as the rate of acetyl-CoA formation (mmol min (kg wet liver tissue)) using methodology described previously by (Cederblad et al. 1990).

**RNA Extraction.** Total RNA was extracted from 30–50 mg of frozen 'wet' liver tissue using TRIzol reagent (Invitrogen, Paisley, UK). Aliquots of RNA were assessed for purity and quantification was verified using a Nanodrop ND-100 (Thermo Fisher Scientific, Delaware USA). Reverse transcription was then carried out from 500 ng of total RNA using the SuperScript III cDNA kit (Invitrogen, Paisley, UK).

**Real-time quantitative PCR.** Taqman primers and probes sets were obtained from Applied Biosystems, UK. Real-time PCR was performed using PCR Universal Master Mix (Applied Biosystems) in a Micro- Amp 96-well plate using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, UK). Assays were performed in triplicate. The threshold (Ct) values for each triplicate were averaged and the quantification of expression of each gene relative to  $\beta$ -actin determined using the standard curve method (Tsintzas, et al. 2006).

**Protein extraction.** Protein was extracted from the organic phase of the RNA extraction homogenate solution. Briefly, 1.5ml of isopropanol per ml of Trizol originally used was added to each sample. Samples were mixed, and left at room temperature for 10 minutes to allow for protein precipitation. Samples were centrifuged at 12,000g for 10 minutes, 2ml of wash solution was added and samples were mixed on a daisy wheel for 20 minutes at room temperature. Samples were centrifuged at 7,500g for 5 minutes at 4°C, then pellets were vortexed in 2ml of 100% EtOH and left to stand at room temperature for 20 minutes. Samples were then centrifuged at 7,500g for 5 minutes at 4°C. Protein pellets were then re-dissolved in 400 $\mu$ l, of protein re-suspension solution (50 mM Tris, 4% SDS and 9 M Urea) and stored

at -80°C. Quantification of protein concentration in the supernatant from liver tissue was conducted using the Pierce Bovine Serum Albumin (BSA) Protein Assay.

**Western blotting.** Protein separation was carried out using SDS-PAGE, using 5-20% gradient gels and then transferred overnight onto a hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Membranes were then incubated in blocking buffer (e.g., BSA or milk) on a shaker for 1 hour. Primary antibodies for target proteins PDK4 (Santa Cruz Biotechnology Inc., USA, #sc-14495), pACC (Cell Signalling, USA, #3661), PGC1 $\alpha$  (Calbiochem, USA, #516557), PPAR $\alpha$  (Abcam, UK, #8934), SIRT1 (Cell Signalling, USA, #2310) and housekeeping protein cyclophilin-B (Abcam, UK, #74173) were diluted in TBS and blocking buffer (1-5%) applied to membranes that were then incubated overnight at 4°C on a shaker. Following the incubation period, membranes were incubated with rabbit anti-goat, goat anti-mouse and swine anti-rabbit HRP (Dako, Denmark) secondary antibodies as appropriate at ratio of 1:2000 diluted in TBS-T containing 1-2% blocking buffer, for 1hr at room temperature. All immunoreactive proteins were visualized using ECL plus (Amersham Biosciences, UK) and quantified by densitometry using the Quantity One 1-D Analysis Software version 4.5 (Bio-Rad Laboratories, Inc., USA).

**Radiotelemetry.** An additional study was carried out with ~4 month old adult male Siberian hamsters that had been housed in LD since birth (n=12). Core body temperature and activity were measured using radiotelemetry devices (TA10TA-F20) and receivers (RPC-1) and ARTv2.1 software (all Data Sciences International, St Paul, MN, USA). Each hamster was implanted with a telemetry device under general anaesthesia as previously reported (Murphy et al, 2012). After a 7-day recovery period, recordings were made for a period of 7 days to establish baseline parameters. Hamsters were then implanted under general anaesthesia with mini-osmotic pumps as described above for the main study. These delivered vehicle (n=6) or FGF21 (n=6) at a dose of 3 mg/kg BW/day. Body weight was recorded daily, and telemetry recordings were made on days 3-4 and days 5-6 after the start of treatment.

**Statistical analysis.** Descriptive statistics (mean  $\pm$  S.E.M) were generated using Graphpad Prism (Prism 6.0, GraphPad, San Diego, CA). After checking for normality of distribution and equality of variance, body weight, food intake and CLAMS data were analysed using a two-way (treatment x sampling time) repeated measures ANOVA with Bonferroni correction used as a post-hoc test. Tissue weights, metabolite analysis, PDC activity, gene expression analysis and protein abundance were analysed using one-way ANOVA or student unpaired t-test where appropriate. No animals were excluded from the analysis. Statistical significance was declared at  $P < 0.05$ .

## Results

**Body weight, food intake and tissue weights.** In animals chronically treated with FGF21 there was a progressive decrease in body weight during the first 6 days of the infusion period, after which body weight became relatively stable (Fig 1A). At the end of the 14-day treatment period body weight was decreased by 10% ( $P < 0.05$ ) in animals treated with FGF21 when compared to vehicle-treated controls at the end of the 14-day treatment period (Fig. 1A). There were no significant effects of chronic FGF21 administration on daily food intake (Fig. 1B). There were 24% and 10% decreases in the weight of the epididymal white adipose tissue ( $P < 0.01$ ) and liver ( $P < 0.05$ ) weights, respectively, following 14-days of the FGF21 infusion when compared to those of vehicle-treated controls.

**Whole-body energy expenditure and carbohydrate and fat oxidation rates.** There was clear circadian variation in metabolic fuel oxidation in animals treated with vehicle, with COX increasing in the dark phase when animals tend to be more active and eat more, and FOX being lowest in the light phase when animals tend to be less active and food intake is reduced (Fig. 2). There was a significant ( $P < 0.05$ )

increase in daily energy expenditure in animals chronically treated with FGF21 when compared to that of vehicle (Fig. 2A). Furthermore, there were clear effects of FGF21 administration on carbohydrate and fatty acid oxidation by day 8 of the infusion period. Specifically, there was a significant reduction (9%,  $P<0.001$ ) in carbohydrate oxidation (Fig. 2B) with a reciprocal increase (58%,  $P<0.001$ ) in fatty acid oxidation (Fig. 2C).

Following the 14-day infusion period there was approximately a 40% decrease ( $P<0.05$ ) in hepatic PDC activity in animals treated with FGF21 when compared to that of vehicle-treated controls (Fig. 3A). In line with the decrease in PDC activity, there was approximately a 34% ( $P<0.05$ ) decrease in hepatic acetylcarnitine accumulation, an index of PDC flux, in animals treated with FGF21 when compared to that of vehicle treated controls (Fig. 3B). There was also a significant reduction ( $P<0.05$ ) in hepatic triglyceride content, which was decreased on average by approximately 47% in animals treated with FGF21 when compared to vehicle treated controls following 14 days of the treatment period (Fig. 4A). Furthermore, in line with the decreased hepatic triglyceride content, there was a 35% ( $P<0.05$ ) increase in hepatic long-chain acylcarnitine accumulation in animals treated with FGF21 when compared to that of vehicle treated control animals (Fig. 4B).

**Hepatic mRNA and protein expression.** In order to identify the potential molecular mechanisms by which FGF21 may be regulating hepatic lipid and carbohydrate metabolism, mRNA and protein analysis was conducted. Following 14 days of the infusion period, there were clear FGF21-induced effects on hepatic mRNA and protein expression of several known regulators of carbohydrate and fatty acid metabolism. FGF21 treatment resulted in a 1.8-fold increase in PDK4 ( $P<0.05$ , Fig. 3C), 2-fold increase in pACC ( $P<0.05$ , Fig. 4C) and 1.6-fold increase in sirtuin 1 (SIRT1, Fig. 5) protein abundance when compared to vehicle treated animals. In animals treated with FGF21 there was a trend towards increased mRNA expression of the known transcriptional regulators of carbohydrate and fat metabolism

PPAR $\alpha$  (P=0.08, Fig 6A) and PGC1 $\alpha$  (P=0.10, Fig. 6C). In line with these effects there was a 1.8-fold (P<0.05) increase in hepatic protein abundance of PGC1 $\alpha$  in animals treated with FGF21 when compared to vehicle (Fig. 6B), but there was no significant effect on PPAR $\alpha$  protein content (Fig 6D).

**Body temperature and locomotor activity.** In order to identify whether weight loss might be a reflection of increased thermogenesis or increased physical activity, core body temperature and locomotor activity were monitored by radiotelemetry in a separate group of hamsters that were being infused with 3 mg/kg/day FGF21. This treatment induced significant weight loss as compared to the vehicle-infused control group (Fig. 7 top, treatment x time interaction F=16.2, P<0.0001). Core body temperature showed clear nocturnal-diurnal variation in both vehicle- and FGF21-treated hamsters (Fig. 7 middle), but there were no significant effects of FGF21 treatment on mean, maximum, or minimum body temperature at either 3-4 days or 5-6 days after the start of treatment. Likewise, both vehicle- and FGF21-treated hamsters displays clear daily variation in their activity patterns (Fig. 7 bottom), but despite some evidence of increased variation in activity levels between individuals in the FGF21-treated group, there was no significant effect on any parameter of locomotor activity.

## Discussion

The primary objective of this study was to investigate the mechanisms by which FGF21 affects hepatic integration of carbohydrate and fat metabolism in a seasonal animal model of adiposity. Chronic treatment with human recombinant FGF21 led to reduced body weight and fat mass, increased whole-body energy expenditure, and a clear shift in metabolic fuel utilization, with a preferential increase in FOX and decrease in COX. These observations are consistent with previous studies of the effects of FGF21 in other dietary and genetic rodent models of obesity (Coskun et al, 2008; Hale et al, 2012), and by using a lower dose of FGF21 than in our previous study (Murphy et al, 2013) we were able to observe

effects of FGF21 on metabolic physiology that are independent of the suppressive effects of FGF21 on food intake in hamsters. These effects of FGF21 on energy expenditure are unlikely to be secondary to effects on locomotor activity or thermogenesis, as in a second study using radiotelemetry we found no evidence for either increased locomotor activity or increased body temperature. Again such observations are consistent with studies in mice and rats where treatment with FGF21 did not affect activity (Coskun et al, 2008; Sarruf et al 2010), or where genetic modification of mice to produce overexpression of FGF21 in the liver actually decreased nocturnal body temperature (Inagaki et al, 2007) and activity (Bookout et al, 2013).

At the tissue level we have shown for the first time that these whole-body metabolic effects are associated with reduced hepatic PDC activity, reduced acetylcarnitine accumulation, and increased long-chain acylcarnitine content, which collectively indicate a FGF21-induced decrease in hepatic COX with a concomitant increase in FOX. This hepatic shift in metabolic fuel utilisation from CHO towards fat oxidation was underpinned by (a) a PDK4-induced impairment in PDC activation through up-regulation of its transcriptional activator PGC1 $\alpha$ ; and (b) inhibition of ACC, as indicated by increased phosphorylation (deactivation) of the protein. This deactivation can potentially alleviate the ACC-mediated inhibition of carnitine palmitoyl transferase 1 (CPT1), resulting in increased delivery of cytosolic long-chain fatty acids (presumably originating from either adipose tissue and/or the hepatic TG content that decreased by 24% and 47%, respectively, in response to treatment with FGF21) across the mitochondrial membrane for subsequent  $\beta$ -oxidation, as indicated by an increase in hepatic long-chain acylcarnitine content.

The PDC regulates the entry of pyruvate into the TCA cycle for subsequent ATP production, linking the glycolytic degradation of glucose to the TCA cycle through the irreversible decarboxylation of pyruvate into acetyl-CoA (Sugden and Holness 2003). Here, in accordance with the whole-body decrease in COX,

there was a 40% decrease in hepatic PDC activity, with a concomitant 65% decrease in acetylcarnitine accumulation, which may be indicative of decreased hepatic PDC flux (Tsintzas, et al. 2007; Wall, et al. 2011). Importantly, the PDC is subject to both acute and chronic inhibition via PDK4, which phosphorylates and inhibits its activity (Sugden, et al. 2009). We have previously shown that selective upregulation of PDK4 and a concomitant inactivation of PDC in human skeletal muscle is linked to a decrease in insulin-stimulated glucose turnover and oxidation in metabolic states characterised by elevated lipid availability and insulin resistance (Chokkalingam, et al. 2007; Tsintzas et al. 2007). In the present study, in line with the decreased hepatic PDC activity, we report a significant increase in protein abundance of PDK4 in animals treated with FGF21 when compared to vehicle. Since PDC is the rate-limiting step in glucose oxidation, taken together these data indicate that the FGF21-induced suppression of hepatic carbohydrate oxidation may occur via PDK4-mediated suppression of PDC activity. Thus, for the first time here we provide a potential mechanistic explanation for the FGF21 mediated inhibition of hepatic carbohydrate oxidation. We have previously shown in human skeletal muscle that accumulation of tissue lipids in response to increased fat oxidation plays an important role in the induction of PDK4 and impairment of PDC activity (Tsintzas et al. 2007), and therefore it is possible that in the present study accumulation of intrahepatic lipid metabolites (as indicated by an increase in long-chain acylcarnitine content) may be responsible for the up regulation of PDK4 protein content. It is also possible that this FGF21-induced hepatic down regulation of carbohydrate oxidation may be required to facilitate the shift in hepatic fuel utilisation towards fat oxidation and subsequent decrease in hepatic triglyceride content. In support of the latter notion, unpublished data from our laboratory indicates that the FGF21-induced impairment in hepatic PDC activity (mins) occurs before the accumulation of hepatic long-chain acylcarnitine content and inhibition of ACC (hours).

To further investigate the molecular mechanism that may be responsible for the FGF21-induced increase in PDK4 protein abundance, known downstream targets of FGF21 ( $PGC1\alpha$  and SIRT-1) and reported

activators of PDK4 expression were measured (Wende, et al. 2005). At the end of the infusion period there was a significant increase in protein abundance of the key hepatic transcriptional co-regulators of energy metabolism PGC1 $\alpha$  and SIRT-1 in animals treated with FGF21 when compared to control. In the liver, FGF21 has been reported to be capable of directly inducing PGC1 $\alpha$  mRNA expression and protein abundance (Adams et al. 2012; Coskun et al. 2008). Potthoff *et al* have also shown using hepatic specific PGC1 KO mice that this protein is critical to the ability of FGF21 to increase hepatic  $\beta$ -oxidation, ketogenesis and TCA cycle flux (Potthoff et al. 2009). Furthermore, PGC1 $\alpha$  has been shown to be capable of directly inducing PDK4 expression in skeletal muscle and hepatocytes (Attia, et al. 2010; Wende et al. 2005). Collectively, these data suggest that the FGF21-mediated induction of PDK4 may occur via a PGC1 $\alpha$  dependent mechanism, however, further investigation is required to confirm this and elucidate the upstream signalling cascade responsible for this effect.

Hepatic fatty acid metabolism is also subject to acute and chronic regulation via the master enzymatic switch acetyl-CoA carboxylase (ACC), which catalyses the conversion of acetyl-CoA to malonyl-CoA, the essential substrate in fatty acid synthesis but also an allosteric inhibitor of CPT1 (McGarry and Brown 1997). Importantly, ACC is itself subject to inhibition by phosphorylation thereby lowering the production of malonyl-CoA and alleviating its inhibitory effect on CPT1 subsequently increasing mitochondrial fatty acid oxidation (Foster 2012). In the present investigation, there was a significant increase in the phosphorylated (and therefore inhibited) form of ACC in the liver of animals treated with FGF21. Thus, it appears that chronic FGF21 administration may be associated with stable inactivation of hepatic ACC therefore lowering tissue levels of malonyl-CoA and alleviating its inhibitory effect on CPT1, allowing the transport of long-chain fatty acids into the mitochondria for their subsequent  $\beta$ -oxidation. In support of these data, Coskun *et al.* (Coskun et al. 2008) have reported that following 14 days of continuous infusion of FGF21, resistance to diet induced obesity in mice was associated with a substantial reduction in several genes associated with hepatic fatty acid synthesis including ACC.

Interestingly, FGF21 has been shown to be capable of directly inhibiting ACC activity in adipose tissue of mice with the authors proposing that this inhibition occurs via a FGF21 induced activation of the AMPK/ACC/PGC1 $\alpha$ /SIRT1 pathway (Chau, et al. 2010). In support of these data here we report a significant increase in hepatic SIRT-1 protein abundance in animals treated with FGF21 when compared to that of vehicle. Thus, it can be argued that the FGF21-mediated inactivation of ACC may be a result of increased SIRT1 protein abundance and therefore activation (deacetylation) of its downstream targets. However, it is important to note that in addition to the direct effects of FGF21 on hepatic ACC activity it may also be the case that increased delivery of fatty acids into the mitochondria is facilitated via an indirect mechanism due to lower production of acetyl-CoA through the PDC reaction and therefore lower levels of malonyl-CoA production, as indicated by the reduction in hepatic PDC activity and acetylcarnitine accumulation in response to FGF21 in the present study.

In summary, the present study provides novel data indicating that the FGF21-induced reduction in hepatic lipid content is associated with a shift in metabolic fuel utilisation from CHO to fat that is facilitated by (i) hepatic PDK4-induced impairment in PDC activation, the rate-limiting step in glucose oxidation, and (ii) inhibition of ACC, which controls the production of the metabolic inhibitor of CPT1 and hence the delivery of long-chain fatty acids across the mitochondrial membrane for subsequent  $\beta$ -oxidation. Our data also indicate that the FGF21-induced activation of the PGC1 $\alpha$  and SIRT1 pathway may underpin this dual effect of FGF21 on carbohydrate and fat oxidation in the liver. Thus, the data presented in this study provide novel mechanistic support for the notion that FGF21 exerts profound metabolic benefits in the liver by modulating its nutrient flux through the carbohydrate metabolism pathway that facilitates the shift in fuel utilisation towards fat oxidation, and therefore may be an attractive target for protection from increased hepatic lipid content and insulin resistance that frequently accompany obesity and diabetes.

**Declaration of Interest:** The authors have no conflict of interest.

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**Author contributions:** RJS and KT wrote manuscript and researched data; MM, MJF and SC researched data and reviewed manuscript; PE, TC, ACA and AK reviewed/edited manuscript; FJPE designed studies, researched data and reviewed/edited manuscript. PE, TC, ACA and AK are employees of Eli Lilly and Company.

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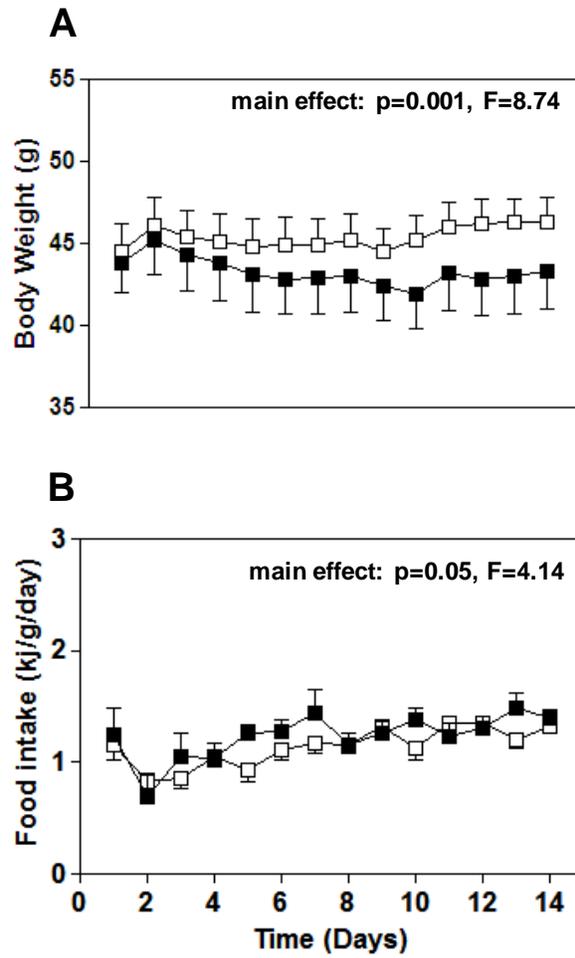
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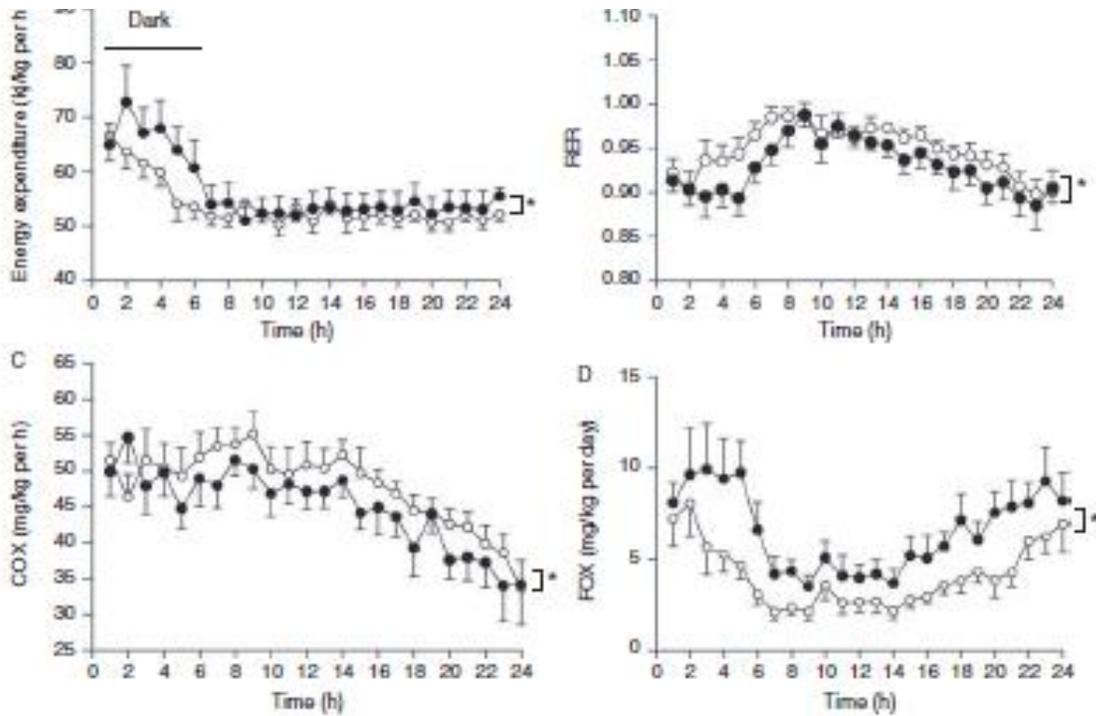
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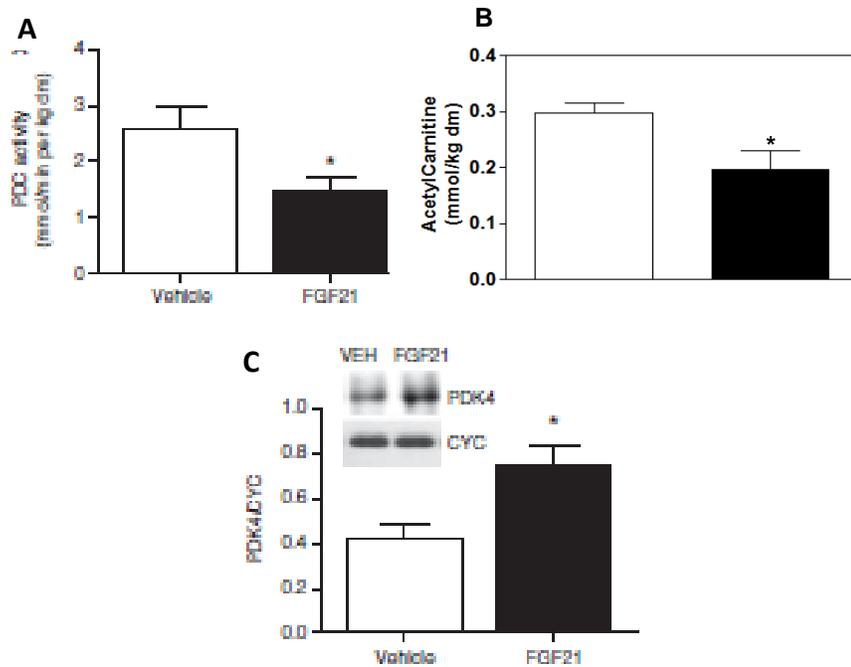
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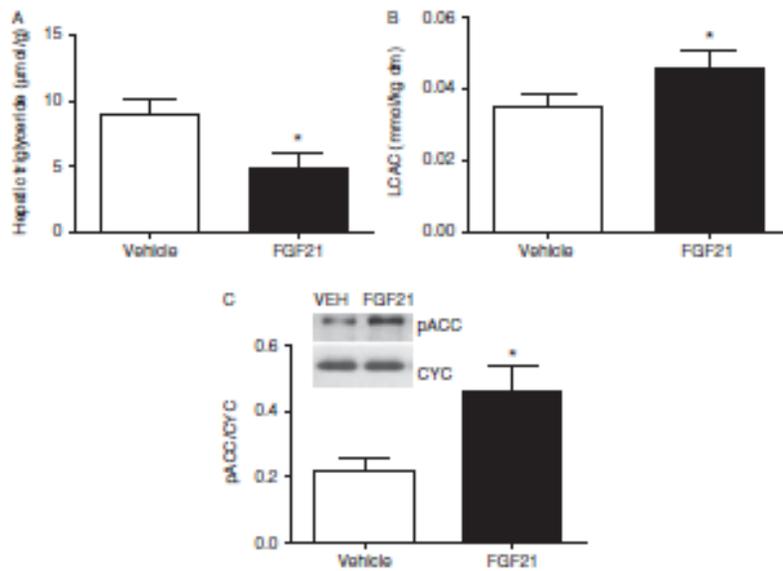
**Figure 1.** The effect of vehicle (n=6) and FGF21 (1 mg/kg/day; n=6) on **(A)** body weight (g/day) and **(B)** food intake (g/day) throughout the 14-day treatment period. Values are group means  $\pm$  S.E.M. \* $P<0.05$  vs vehicle.



**Figure 2.** The effect of vehicle (n=6) and FGF21 (1mg/kg/day; n=6) on **(A)** whole-body energy expenditure (kJ/kg/hr), **(B)** carbohydrate (COX, mg/kg/hr) and **(C)** fat oxidation (FOX, mg/kg/hr) rates for a 24-hour period following 7 days of FGF21 treatment. Values are group means  $\pm$  S.E.M. \*P<0.05 and \*\*P<0.01 treatment effect vs vehicle.

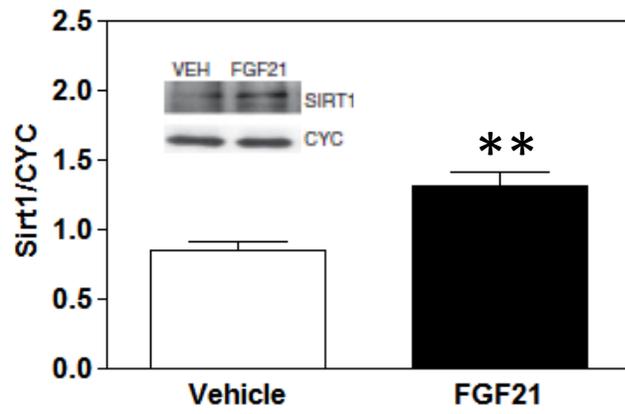


**Figure 3.** The effect of vehicle (n=6) and FGF21 (1 mg/kg/day; n=6) on **(A)** pyruvate dehydrogenase complex activity (PDC, mmol/min/ kg dm), **(B)** acetylcarnitine (mmol/kg dm) and **(C)** pyruvate dehydrogenase 4 (PDK4) at the end of the 14-day treatment period. Values are group means  $\pm$  S.E.M. \*P<0.05 vs vehicle.

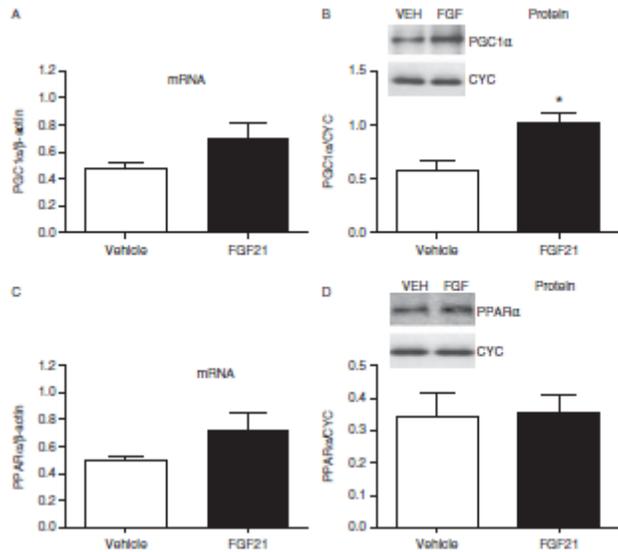


**Figure 4.** The effect of vehicle (n=6) and FGF21 (1 mg/kg/day; n=6) on (A) hepatic triglyceride content (μmol/g), (B) long chain acylcarnitine (LCAC, mmol/kg dm) accumulation and (C), phosphorylated acetyl-CoA carboxylase (pACC) at the end of the 14-day treatment period. Values are group means ± S.E.M.

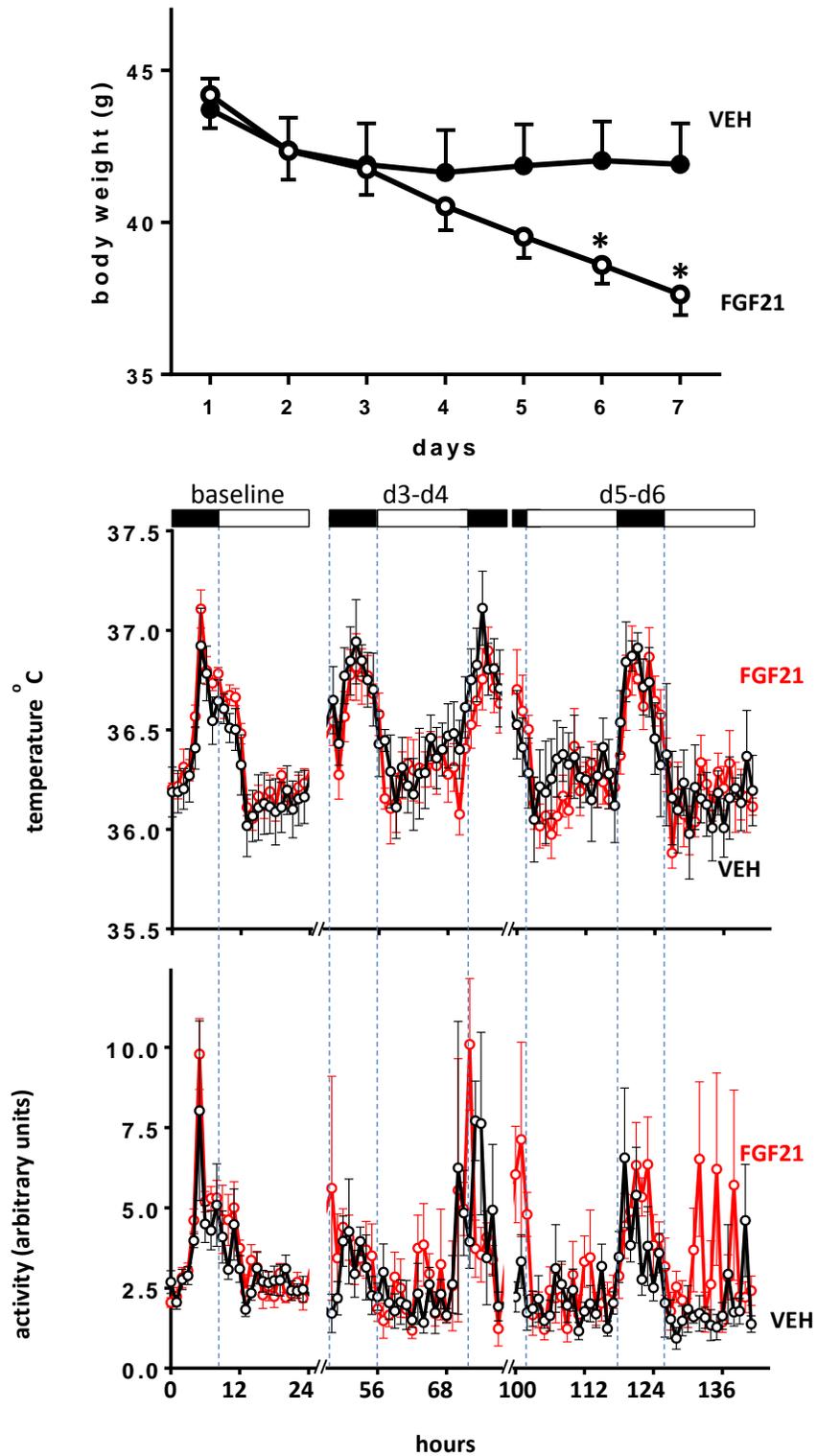
\*P<0.05 vs vehicle.



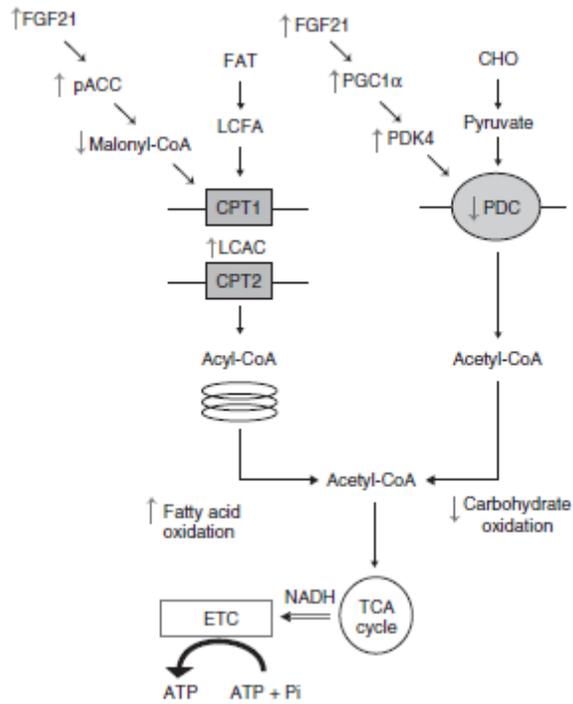
**Figure 5.** The effects of vehicle (saline; n=6) and FGF21 (1 mg/kg/day; n=6) on SIRT 1 protein abundance normalised to cyclophilin-B (CYC) after 14-days of infusion. Values are means  $\pm$  S.E.M. \*\*\*P<0.01 vs vehicle.



**Figure 6.** The effects of vehicle (saline; n=6) and FGF21 (1 mg/kg/day; n=6) on **(A and B)** peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1 $\alpha$ ), and **(C and D)** peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPAR $\alpha$ ) mRNA gene expression normalised to  $\beta$ -actin and protein abundance normalised to cyclophilin- $\Delta$  (CYC) after 14-days of infusion. Values are means  $\pm$  S.E.M. \*P<0.05 vs vehicle.



**Figure 7.** Body weight (top), mean core body temperature (middle) and locomotor activity (bottom) in adult male hamsters infused with vehicle (○, n=6) or recombinant human FGF21 at 3 mg/kg/day (●, n=6). Values are group mean ± SEM for the telemetry variables prior to treatment (baseline), and on day 3 and days 5-6 after the start of treatment, \*P<0.05 vs vehicle. Solid bars depict the 8h dark phase.



**Figure 8.** A schematic diagram of the proposed mechanism(s) by which FGF21 is regulating hepatic fatty acid and carbohydrate metabolism in the Siberian hamster. Fibroblast growth factor 21 (FGF21), phosphorylated acetyl-CoA carboxylase (pACC), pyruvate dehydrogenase kinase 4 (PDK4), pyruvate dehydrogenase complex (PDC), carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2 respectively), long-chain acylcarnitine (LCAC), peroxisome proliferator-activated receptor gamma co-activator 1 (PGC1 $\alpha$ ), increased activity/content (  $\uparrow$  ), reduced activity/content (  $\downarrow$  ).