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Fibroblast growth factor-21 (FGF21) administration to early-lactating dairy cows. II. Pharmacokinetics, whole-animal performance, and lipid metabolism

L. S. Caixeta,[1]* S. L. Giesy,[1]* C. S. Krumm,[1] J. W. Perfield 2nd,[2] A. Butterfield,[2] and Y. R. Boisclair[1]†
[1]Department of Animal Science, Cornell University, Ithaca, NY 14853
[2]Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285

ABSTRACT

Dairy cows cope with severe energy insufficiency in early lactation by engaging in intense and sustained mobilization of fatty acids from adipose tissue. An unwanted side effect of this adaptation is excessive lipid accumulation in the liver, which in turn impairs hepatic functions. Mice experiencing increased hepatic fatty acid flux are protected from this condition through coordinated actions of the newly described hormone fibroblast growth factor-21 (FGF21) on liver and adipose tissue. The possibility of an analogous role for FGF21 in dairy cows is suggested by its rapid increase in plasma levels around parturition followed by chronically elevated levels in the first few weeks of lactation. To test this hypothesis, dairy cows were randomly assigned on d 12.6 ± 2.2 (± standard error) of lactation to receive either an excipient (control; n = 6) or recombinant human FGF21 (n = 7), first as an FGF21 bolus of 3 mg/kg of body weight (BW) followed 2 d later by a constant i.v. infusion of FGF21 at a rate of 6.3 mg/kg of metabolic BW for 9 consecutive days. After bolus administration, human FGF21 circulated with a half-life of 194 min, and its constant infusion increased total plasma concentration 117-fold over levels in excipient-infused cows. The FGF21 treatment had no effect on voluntary feed intake, milk yield, milk energy output, or net energy balance measured over the 9-d infusion or on final BW. Plasma fatty acids circulated at lower concentrations in the FGF21 group than in the control group for the 8-h period following bolus administration, but this reduction was not significant during the period of constant i.v. infusion. Treatment with FGF21 caused a 50% reduction in triglyceride content in liver biopsies taken at the end of the constant i.v. infusion without altering the mRNA abundance of key genes involved in the transport, acyl coenzyme A activation, or oxidation of fatty acids. In contrast, FGF21 treatment ablated the recovery of plasma insulin-like growth factor-1 seen in control cows during the 9-d i.v. infusion period despite a tendency for higher plasma growth hormone. This effect was associated with increased hepatic mRNA abundance of the intracellular inhibitor of growth hormone receptor trafficking, *LEPROT*. Overall, these data confirm the ability of FGF21 to reduce lipid accumulation in bovine liver and suggest the possibility that FGF21 does so by attenuating the hepatic influx of adipose tissue-derived fatty acids.
Key words: β-hydroxybutyrate, fatty acid, growth hormone resistance, insulin-like growth factor-1, liver

INTRODUCTION

Dairy cows experience a severe energy insufficiency in early lactation as a consequence of mammary nutrient demands occurring in the absence of adequate compensatory feed intake (Bell, 1995; Drackley et al., 2001; Boisclair et al., 2006). Dairy cows close the energy gap in part by engaging in intense and sustained mobilization of fatty acids from adipose tissue. This is indicated in the first few days of lactation by peaks of plasma fatty acids in the range of 600 to 1,000 μ*M* reflecting daily mobilization rates of 2.2 to 3.7 kg (Drackley et al., 2001) and by sufficient amounts of fatty acids mobilized over the first month of lactation to cover 30% of total energy requirements (Bell, 1995; Drackley et al., 2001). One drawback of accelerated fatty acid mobilization, however, is ectopic lipid accumulation, with the liver being particularly vulnerable to this condition (Bobe et al., 2004; Geelen and Wensing, 2006). Liver susceptibility to lipid accumulation reflects its ability to extract 20 to 25% of incoming fatty acids irrespective of circulating concentrations (Drackley et al., 2001). With the elevated fatty acid concentration of early lactation, hepatic uptake far exceeds disposal by oxidation and secretion as very low density lipoprotein (**VLDL**; Drackley et al., 2001). The moderate to severe lipidosis that follows not only impairs hepatic functions such as gluconeogenesis but also is negatively associ-

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*These authors contributed equally to this work.
†Corresponding author: yrb1@cornell.edu

ated with whole-animal outcomes such as reproduction (Rukkwamsuk et al., 1999; Geelen and Wensing, 2006).

The identification of excessive lipid mobilization and insufficient hepatic disposal as root causes of liver lipidosis has prompted efforts to identify hormones involved in their regulation. This work has implicated low plasma insulin and growth hormone (**GH**)–dependent potentiation of adipose tissue response to catecholamines as drivers of fatty acid mobilization in early lactation (Vernon, 2005; Boisclair et al., 2006) and has shown that glucagon therapy limits liver triglyceride accumulation (Nafikov et al., 2006). Work in rodents has recently implicated fibroblast growth factor-21 (**FGF21**) as another potential important regulator of fatty acid metabolism (Fisher and Maratos-Flier, 2016; BonDurant and Potthoff, 2018). Fibroblast growth factor-21 belongs to the fibroblast growth factor (**FGF**) superfamily of growth factors, but, unlike most other members, it lacks the high-affinity heparin domain needed for FGF retention through proteoglycan binding (Itoh et al., 2016). Therefore, FGF21 can leave the site of synthesis and act as a hormone. In the mouse, virtually all circulating FGF21 is produced in the liver, where production depends on the presence of the transcription factors peroxisome proliferator-activated receptor-α (**PPARA**) and cAMP responsive element binding protein 3-like 3 (**CREB3L3** or **CREBH**) and the deacetylase sirtuin 1 (**SIRT1**; Fisher and Maratos-Flier, 2016). Mice lacking these regulatory proteins develop more severe steatosis than their wild-type counterparts when fasted or offered high-fat diets, and this condition can be prevented by exogenous FGF21 therapy (Inagaki et al., 2007; Li et al., 2014; Park et al., 2016). Mechanistic studies have traced the reduction of liver steatosis by FGF21 to stimulation of fatty acid disposal in the liver and repression of lipolysis in adipose tissue (Arner et al., 2008; Li et al., 2009; Potthoff et al., 2009; Fisher et al., 2014).

The possibility that a single hormone could limit hepatic lipid accumulation through coordinated actions on adipose tissue and liver is highly relevant to early-lactating dairy cows. In our previous work, we uncovered a dynamic increase in plasma FGF21 at the onset of lactation and identified liver as its primary source (Schoenberg et al., 2011). More recently, we implicated plasma fatty acids as a key factor driving hepatic FGF21 production and increasing circulating FGF21 in cattle (Caixeta et al., 2017). At this time, however, no information is available on the role of FGF21 in cattle and, more specifically, on the possibility that it limits hepatic accumulation of lipids in early lactation. Our results indicate that exogenous FGF21 reduces the accumulation of triglyceride in liver and does so in the absence of detrimental effects on performance.

MATERIALS AND METHODS

Animals and Design

All procedures were conducted with the approval of the Cornell University Institutional Care and Use Committee. Thirteen Holstein dairy cows of uniform parity (2.3 ± 0.2; ±SE) and body condition (3.5 ± 0.2) were selected. Cows were moved on d 9.6 ± 2.2 of lactation to individual stalls under a controlled environment (20°C, light on 0500–2100 h) and fitted with bilateral intrajugular catheters (Tygon S-54-HL, 1.02-mm i.d. × 1.78-mm o.d.; Saint-Gobain Performance Plastics, Akron, OH). They were offered nonlimiting amounts of a dry TMR (Table 1) every 2 h using in-house automatic feeders and were milked twice daily at 0700 and 1700 h.

Cows were then randomly allocated to an excipient solution (control; n = 6) or recombinant human FGF21 (n = 7) and studied in 2 consecutive phases. On d 11.6 ± 2.2, basal blood samples were obtained every 2 h between 0800 and 1200 h followed by measurement of BW (control = 636.0 ± 24.0 kg; FGF21 = 648.0 ± 24.9 kg). In the first phase, treatments were administered as

Table 1. Ingredient and nutrient composition of the diet

Item	% of DM
Ingredient	
Alfalfa hay	31.44
Grass hay	9.44
Corn grain, finely ground	23.17
Corn meal	9.80
Distillers grains	6.16
Wheat middlings	5.91
Soybean hulls	5.68
Soybean meal, cooker processing[1]	1.96
Canola meal	1.96
Molasses	1.43
Bypass fat	0.91
Sodium bicarbonate	0.69
Salt	0.40
Calcium carbonate	0.35
Blood meal	0.31
Urea	0.22
Magnesium oxide, 54%	0.05
Selenium, 0.06%	0.04
Vitamin A, D, E premix[2]	0.03
Rumensin 90[3]	0.01
Chemical analysis	
CP	18.06
ADF	21.60
NDF	34.73
Starch	20.98
Crude fat	3.80
Ash	7.08
Ca	0.96
P	0.40

[1]AminoPlus (Ag Processing Inc., Omaha, NE).

[2]Contained 30,464 IU/kg vitamin A, 5,862 IU/kg vitamin D, and 93,784 IU/kg vitamin E.

[3]Elanco Animal Health (Greenfield, IN).

a single intrajugular bolus at 0800 h on d 12.6 ± 2.2 of lactation. The FGF21 dose was 3 mg/kg of BW and was selected on the basis of positive responses when given as a single injection in the mouse (Xu et al., 2009b). The dose was prepared with the human FGF21 variant LY2405319 with 78% identity with mature bovine FGF21 and bioactivity in cattle (Krumm et al., 2019); LY2405319 was engineered for enhanced in vivo stability and to retain full biological activity (Adams et al., 2013; Kharitonenkov et al., 2013). It was produced in *Pichia pastoris*, purified to homogeneity using reversed-phase and anion-exchange chromatography, and prepared as a 33 mg/mL solution in an excipient solution of 10 m*M* citrate, 150 m*M* NaCl, pH 7.0 (Eli Lilly, Indianapolis, IN; Kharitonenkov et al., 2013). The LY2405319 solution was kept frozen until use, as done in previous studies in humans and monkeys (Adams et al., 2013; Gaich et al., 2013). Blood samples were obtained at fixed times relative to bolus administration (−60, −30, 0, 1, 5, 10, 20, 40, and 60 min and 1.5, 2, 3, 4, 6, 8, 10, and 12 h), immediately mixed with heparin (15 IU/mL), and centrifuged at 3,000 × *g* for 15 min at 4°C. Resulting plasma was stored at −20°C until being analyzed for plasma FGF21 and fatty acids.

The second phase was initiated 2 d later at 0800 h and involved continuous intrajugular infusion of either excipient or FGF21 solution for 9 consecutive days. The daily FGF21 dose was 6.3 mg/kg of metabolic BW and was selected on the basis of positive responses when given chronically in rhesus monkeys (Adams et al., 2013). The LY2405319 was prepared in an excipient solution as described above, and both the excipient and LY2405319 solutions were infused at the fixed rate of 5 mL/h using a syringe pump. Blood was collected every 2 h between 0800 and 1200 h on d 1, 3, and 8 of infusion and at 0800 h on other days. An i.v. glucose tolerance test (**GTT**) was performed at 1400 h on d 8 of the chronic infusion. The glucose dose was 0.25 g/kg of BW administered as a dextrose solution (50% wt/vol; VetOne, Boise, ID). Blood samples were collected at −60, −30, −15, −5, 5, 10, 20, 30, 60, 90, and 120 min relative to administration of the glucose bolus. All blood samples collected during this phase were processed to plasma as described above. Finally, a liver biopsy was obtained at the end of chronic infusion on d 9 followed by measurement of BW. The biopsy was obtained after surgical preparation and local anesthesia via percutaneous puncture with a trocar as previously described (Giesy et al., 2012; Caixeta et al., 2017). Biopsies were snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Variables measured at the start and end of treatment included BW and body condition as previously described (Block et al., 2001). Variables measured throughout the treatment period included daily feed intake and milk weight and composition at every milking. The nutrient and chemical composition of the TMR was determined using wet chemistry analysis, and the composition of milk was determined using infrared analysis (Dairy One Cooperative Inc., Ithaca, NY). Equations published by NRC (2001) were used to calculate the TMR energy content and milk energy output. Individual energy intake and milk energy output averaged over the entire treatment period were used to calculate net energy balance as described previously (Block et al., 2001).

Analysis of Metabolites and Hormones

Plasma fatty acids and BHB were analyzed using spectrophotometric methods based on the enzymes acyl coenzyme A–oxidase and BHB dehydrogenase, respectively (Caixeta et al., 2017). The plasma fatty acid response area during the GTT was calculated between 0 and 120 min and was corrected for baseline concentrations (mean of concentrations at −60, −30, −15, and −5 min; Sechen et al., 1989; Ehrhardt et al., 2001). Total and intact FGF21 were determined using an in-house sandwich FGF21 ELISA differing only in the antibody used for detection (rabbit polyclonal against human FGF21 for total FGF21; mouse monoclonal against the C terminal FGF21 region for intact FGF21) and validated in bovine plasma (Schoenberg et al., 2011; Caixeta et al., 2017). Growth hormone and IGF-1 were measured using in-house RIA validated in bovine plasma (Block et al., 2001; Leury et al., 2003). Inter- and intra-assay coefficients of variation were <10 and <9%, respectively, for metabolite assays and <9 and <9%, respectively, for hormone assays.

Liver triglycerides were measured using a method validated in cattle involving extraction of total lipids with the Folch procedure followed by determination of triglycerides using the colorimetric Hantzsch condensation method (Schoenberg et al., 2011; Caixeta et al., 2017). Total levels of ceramides and diacylglycerol in liver were measured using methods we previously described (Estridge et al., 2017; Pillon et al., 2018). In brief, liver samples were homogenized in a Qiagen (Valencia, CA) TissueLyzer II followed by addition of an internal standard mixture consisting of ^{15}N,$^{13}C3$-ceramide-16:0 and diacylglycerol 15:0/15:0 (Eli Lilly). Lipids were isolated using 2-phase extraction of aqueous methanol and chloroform. Lipidomics was conducted via flow injection electrospray ionization-tandem MS using a 5600 TripleTOF system (AB Sciex, Framingham, MA). The mass spectra were acquired in 2 stages. In the first stage, the time of flight spectra were scanned with minimal collision energy from 100 to 1,200 Da with high resolution. The second stage consisted of time of

flight product ion scans of 611 precursor masses from 349.199 to 959.809 isolated with a resolution of 0.7 Da and changed stepwise in 1.001-Da increments. Lipids were identified in the second stage by precursor and product ion pairs predicted by the analyte species and lipid class. Levels of individual ceramide and diacylglycerol species were quantified by the ratio of analyte to internal standard and summed to obtain total levels of diacylglycerol and ceramide.

Pharmacokinetic Analysis of Human FGF21

The concentrations of total plasma FGF21 in post-bolus samples were analyzed using noncompartmental analyses with a commercial software (PK Solutions 2.0, Summit Research Services, Montrose, CO). This software uses a curve-stripping procedure to resolve the concentration–time curves into a series of exponential terms that correspond to the kinetic phases of this hormone in circulation. The initial total FGF21 concentration was estimated through linear extrapolation of the first 2 plasma concentration values to time zero. Area under the concentration–time curve from 0 to 12 h ($AUC_{0–12\ h}$) was estimated using the trapezoid method up to the last measured concentration at 12 h post-bolus. The apparent terminal disposition rate constant (λ_z) was determined using linear regression analysis of the terminal portion of the log plasma concentration–time curve. Elimination half-life ($t_{1/2}$) and clearance rate were calculated as ln (2)/λ_z and dose/$AUC_{0–12\ h}$, respectively.

RNA Extraction and Analysis of Gene Expression

Liver samples were lysed with Qiazol (Qiagen) followed by total RNA purification using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen). Quality of RNA was determined using the RNA Nano Lab Chip kit and bioanalyzer (Agilent, Palo Alto, CA) with all samples having an RNA integrity number >7.5. Reverse transcription reactions were performed with 2 μg of total RNA in a total volume of 20 μL with the high-capacity cDNA reverse transcription kit and RNase inhibitor (Applied Biosystems, Foster City, CA). Gene expression was analyzed using quantitative real-time PCR using Power SYBR Green Mix (Applied Biosystems). Real-time PCR assays were performed in duplicate with a total 25-μL reaction volume containing 500 n*M* concentration of each primer and reverse-transcribed RNA (25 ng except 2.5 ng for the internal standard gene *18S*). The sequences of all primers used are given in Supplemental Table S1 (https://doi.org/10.3168/jds.2019-16696). Primer pairs were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For all genes, each member of the primer pair was located in adjoining exons and shown by blast analysis to anneal exclusively to the target sequence. For all primer pairs, amplification was greater than 95% efficient and yielded a single product as shown by melting curve analysis. Data were analyzed using a relative standard curve based on a serial 2-fold dilution of pooled cDNA prepared from liver and normalized to the geometric mean of *18S* and *B2M*.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Data collected at a single time point or collated over the treatment period were analyzed using a model accounting for treatment (control vs. FGF21) as fixed effect and cow as the random effect. Data collected over time were analyzed using repeated measures, and the model accounted for treatment (control vs. FGF21) and time as fixed effects and cow as the random effect. Cow was subjected to 6 covariance structures (first-order autoregressive, heterogeneous first-order autoregressive, compound symmetry, heterogeneous compound symmetry, first-order antedependence, and Toeplitz). The structure yielding the smallest Akaike information criterion was selected. A covariate corresponding to data collected immediately before bolus was included in this model. Statistical significance and tendency were respectively set at $P < 0.05$ and $P < 0.10$ for main effects and at $P < 0.10$ and $P < 0.15$ for the FGF21 × Time interactions.

RESULTS

Plasma Profile of Human FGF21 During Acute and Chronic Infusion

On d 12.6 ± 2.2 of lactation, dairy cows received a single i.v. bolus of excipient or 3 mg of human FGF21/kg of BW followed by blood sampling over the next 12 h. The plasma concentration of total FGF21 before bolus in these cows was 5.8 ± 1.7 ng/mL and remained at this level in cows receiving the excipient bolus. In contrast, the plasma concentration of total FGF21 reached 42,150 ng/mL within 5 min in the FGF21 group, followed by a steady decline to 179 ng/mL after 12 h (Figure 1A). Analysis of the plasma total FGF21 concentration–time curve predicted a maximal concentration of 45,275 ng/mL at time 0 and an average elimination $t_{1/2}$ of 194 min (Table 2). Additional kinetic parameters are given in Table 2.

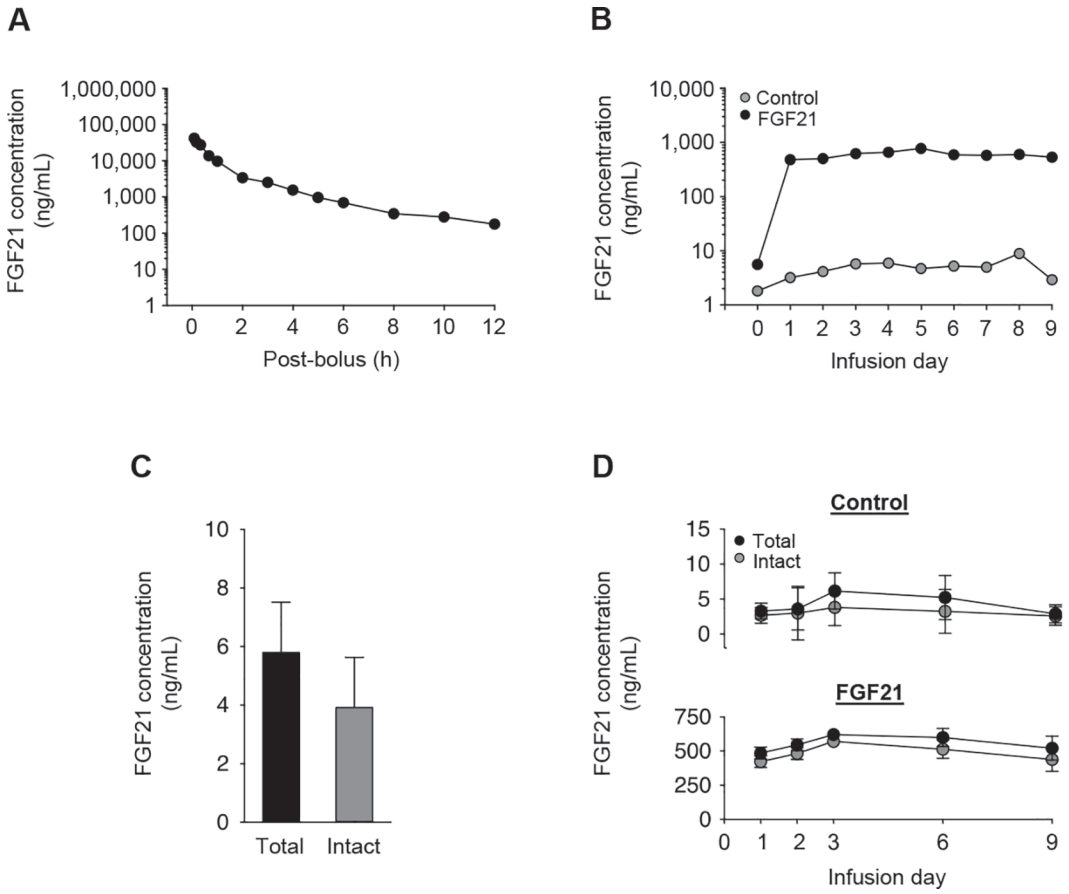

Figure 1. Pharmacokinetics of fibroblast growth factor-21 (FGF21) in early-lactating dairy cows. An excipient (control) or a human FGF21 solution were given i.v. as boluses on d 12.6 ± 2.2 of lactation (FGF21 dose = 3 mg/kg of BW) and as constant infusions (FGF21 dose = 6.3 mg/kg of metabolic BW per day) starting on d 14.6 ± 2.2. Blood samples were collected over the 12-h period following bolus administration and daily over the 9-d constant infusion, and plasma was analyzed for FGF21 concentration. (A) Plasma FGF21 concentration after FGF21 bolus administration. Data shown are plasma FGF21 concentrations corrected for basal concentration measured immediately before bolus administration (5.6 ± 1.3). Each point represents the LSM ± SE of 7 cows. The pooled SE was 1,421 ng/mL. (B) Plasma FGF21 concentration during the 9-d constant infusion period. Each point represents the LSM of 6 to 7 cows. The pooled SE was 1.5 ng/mL for the control group and 27.3 ng/mL for the FGF21 group. (C) Total and intact plasma FGF21 concentrations during the pretreatment period. Each bar represents the LSM ± SE of 13 cows. (D) Total and intact plasma FGF21 concentrations during the 9-d constant infusion period. Each point represents the LSM ± SE of 6 to 7 cows.

Treatments were resumed 2 d later via constant i.v. infusion. Infusion of FGF21 led to a plasma concentration of total FGF21 of 486 ± 39.8 ng/mL after 1 d of infusion and an average concentration of 597 ± 27.3 ng/mL over the 9-d infusion (Figure 1B). This average FGF21 concentration represents a 117-fold increase over the plasma FGF21 concentration of 5.1 ± 1.5 ng/mL measured in the control group over the same period.

Human FGF21 is susceptible to inactivation through proteolytic cleavage at its carboxyl-terminal end (Hecht et al., 2012). To determine whether this processing occurs in cattle, plasma was reassayed, with the intact ELISA detecting only FGF21 with an intact carboxyl-terminal end. As shown in Figure 1C, the intact and total FGF21 assays yielded comparable plasma concentrations during the pretreatment period. Similarly, plasma FGF21 concentrations measured during the treatment period did not differ significantly between intact and total FGF21, including in cows receiving the human FGF21 infusion (Figure 1D).

Table 2. Pharmacokinetic parameters of fibroblast growth factor-21 (FGF21) after a single bolus injection[1]

Parameter[2]	Average ± SE
C_i (ng/mL)	45,275 ± 4,156
$AUC_{0-12\,h}$ (ng·min·mL^{-1})	2,324,850 ± 175,543
Cl (mL·min^{-1}·kg^{-1})	1.3 ± 0.1
Elimination $t_{1/2}$ (min)	194 ± 29

[1]Dairy cows received a single i.v. bolus of human FGF21 (3 mg/kg) on d 12.6 ± 2.2 of lactation (n = 7).

[2]C_i = initial concentration; $AUC_{0-12\,h}$ = area under the concentration–time curve from 0 to 12 h; Cl = clearance rate; $t_{1/2}$ = half-life.

Increased Plasma FGF21 Has No Effect on Performance in Early Lactation

Fibroblast growth factor-21 had no effect on the profile of feed intake, milk yield, or milk energy output over the 9-d infusion period (Figure 2A). A similar lack of FGF21 effect was seen on milk production expressed as FCM and on milk components, with the exception of a higher milk protein content in FGF21-treated cows (Table 3). The net energy balance during the treatment period was similarly negative at −15.7 and −14.9 Mcal/d for control and FGF21 cows, respectively (Figure 2B). Consistent with a lack of effect on energy balance, final BW and BCS did not differ between treatments (Table 3).

Effect of Increased Plasma FGF21 on Lipid Metabolism

Administration of FGF21 to rodents and primates reduces various lipid fractions in plasma, including fatty acids (Li et al., 2009; Xu et al., 2009a; Gaich et al., 2013). To determine whether similar effects were seen in early-lactating dairy cows, plasma fatty acids were analyzed in samples collected during the 12 h following bolus and during the 9-d infusion. Following bolus administration, plasma fatty acids circulated at lower concentrations in the FGF21 group than in the control group for most of the 12-h sampling period (Figure 3A; FGF21 × Time, $P = 0.047$). As expected, plasma fatty acids declined over the 9-d infusion, but the fatty acid–reducing effect of FGF21 was no longer significant (Figure 3B; Time, $P = 0.004$). A statistical tendency was observed for plasma BHB, with numerically lower concentrations in the FGF21 group than in the control group between d 2 and 6 of infusion (FGF21 × Time, $P = 0.15$). Finally, we asked whether FGF21 treatment affected the plasma fatty acid profile during a GTT performed on d 8 of infusion. The GTT procedure led to identical increases and profiles of plasma insulin in both groups (Krumm et al., 2019), causing dynamic reductions in plasma fatty acid concentrations over the first 60 min (Figure 3D; Time, $P < 0.001$). However, FGF21 treatment had no effect on either the profile or the response area of plasma fatty acids.

A second major consequence of FGF21 therapy in laboratory animals is a reduction in liver lipids (Inagaki et al., 2007; Xu et al., 2009a; Fisher et al., 2014). Accordingly, we assayed various lipid species in biopsies taken on d 9 of chronic infusion. The FGF21 treatment had no effect on total diacylglycerol and ceramide levels but caused a 50% reduction in triglyceride content (Figure 4A; $P = 0.036$). This effect was not associated with altered expression of genes encoding

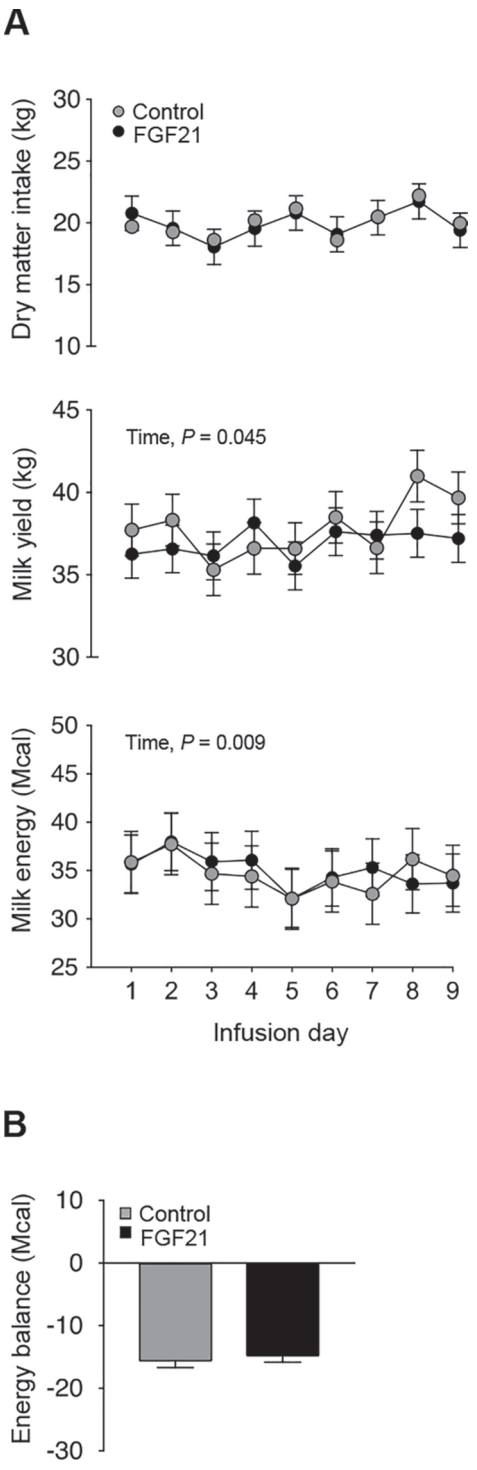

Figure 2. Effect of chronic administration of fibroblast growth factor-21 (FGF21) on production-related variables. Dairy cows received a constant i.v. infusion of an excipient (control) or human FGF21 (6.3 mg/kg of metabolic BW per day) starting on d 14.6 ± 2.2 of lactation and lasting for 9 consecutive days. (A) Dry matter intake, milk yield, and milk energy were measured daily. Each point represents the LSM ± SE of 6 to 7 cows. The significant effect of Time is reported. (B) Net energy balance was calculated for each cow over the 9-d period as the difference between energy intake and output (milk energy and maintenance). Each bar represents the LSM ± SE of 6 to 7 cows.

proteins involved in fatty acid transport (*SLC27A5*), acyl coenzyme A activation of fatty acids (*ASCL1*), or mitochondrial fatty acid oxidation and ketogenesis (*ACADM*, *ACADL*, and *HMGCS2*; Figure 4B).

Effect of Increased Plasma FGF21 on the Hepatic GH–IGF-1 System

Chronic increases in plasma FGF21 in rodents impair hepatic GH action and IGF-1 production (Inagaki et al., 2008; Kubicky et al., 2012). The possibility of similar effects in dairy cows was assessed in plasma samples collected during the basal period and on d 1, 3, and 8 of infusion. The plasma GH concentration decreased over the infusion period in both groups (Figure 5A; Time, $P = 0.005$) but tended to remain elevated in the FGF21 group relative to the control group (FGF21, $P = 0.091$). On the other hand, plasma IGF-1 increased steadily in the control group but remained invariant in the FGF21 group (FGF21 × Time, $P = 0.042$). This effect on plasma IGF-1 was not associated with altered hepatic expression of *IGF1*, the liver-specific GH receptor transcript *GHR1A*, or its signaling inhibitor *SOCS2* (Figure 5B). In contrast, FGF21 treatment increased hepatic expression of the inhibitor of GH receptor trafficking, *LEPROT* (Figure 5B; FGF21, $P = 0.02$).

DISCUSSION

Mobilization of lipid reserves after parturition makes the coexistence of productivity and energy insufficiency possible in high-yielding dairy cows, but it also can be maladaptive (Bell, 1995; Drackley et al., 2001). This is indicated by the persistence of excessively elevated concentrations of fatty acids in plasma followed by their accumulation in the form of triglyceride in nonadipose tissue (Drackley et al., 2001; Bobe et al., 2004). Triglyceride accumulation is particularly detrimental in the liver, where it impairs essential functions such as gluconeogenesis and promotes the synthesis of drivers of peripheral insulin resistance such as ceramides (Rukkwamsuk et al., 1999; McFadden and Rico, 2019). Hepatic lipidosis is also associated with postparturient disorders, including subclinical and clinical ketosis and poor reproductive performance (Drackley et al., 2001; Geelen and Wensing, 2006). Exogenous FGF21 therapy attenuates lipid mobilization and hepatic lipidosis in rodents (Xu et al., 2009a; Li et al., 2014; Park et al., 2016), but it was unknown at the start of this work whether these actions would take place in energy-deficient, early-lactating dairy cows and whether they would occur without negative effects on performance.

We addressed these questions by treating dairy cows with LY2405319, a human FGF21 variant retaining full in vivo activity (Adams et al., 2013; Kharitonenkov et al., 2013); LY2405319 is identical to native human FGF21 except for the removal of the first 4 N-terminal AA (HPIP) and the introduction of the point mutations L118C, A134C, and S167A to improve production in yeast (Kharitonenkov et al., 2013). Human FGF21 was available in the gram amounts needed for work in dairy cows, whereas bovine FGF21 was commercially available only in microgram amounts. This variant is bioactive in a range of species, including rhesus monkeys and mice (Adams et al., 2013; Kharitonenkov et al., 2013). Moreover, it produces metabolic responses in the mouse that are identical to those observed with mouse FGF21 (Kharitonenkov et al., 2013); therefore, it is likely capable of eliciting most if not all of the effects of bovine FGF21. We used this human variant

Table 3. Effect of chronic administration of fibroblast growth factor-21 (FGF21) on final BW, BCS, and milk composition in lactating dairy cows[1]

	Treatment			*P*-value		
Variable	Control	FGF21	SD	FGF21	Time	FGF21 × Time
Final BW[2] (kg)	594.6	586.2	27.0	0.59	—	—
Final BCS[2]	3.0	3.2	0.2	0.32	—	—
Milk[3]						
3.5% FCM[4] (kg)	54.4	52.6	4.3	0.57	0.01	0.80
Lactose (%)	4.9	4.9	0.1	0.68	0.02	0.74
Fat (%)	5.8	5.6	0.8	0.74	0.01	0.99
Protein (%)	2.6	2.8	0.0	0.004	0.06	0.89

[1]Dairy cows received a constant i.v. infusion of excipient (control, n = 6) or human FGF21 (6.3 mg/kg of metabolic BW per day, n = 7) starting on d 15.6 ± 2.2 of lactation and lasting for 9 consecutive days.

[2]Final BW and BCS (1 = thin, 5 = fat) were measured at the end of the infusion period.

[3]Milk components were analyzed at each milking and averaged by day. Values represent the overall mean during the infusion period.

[4]3.5% FCM = 0.4324 × milk kg + (16.218 × milk fat kg).

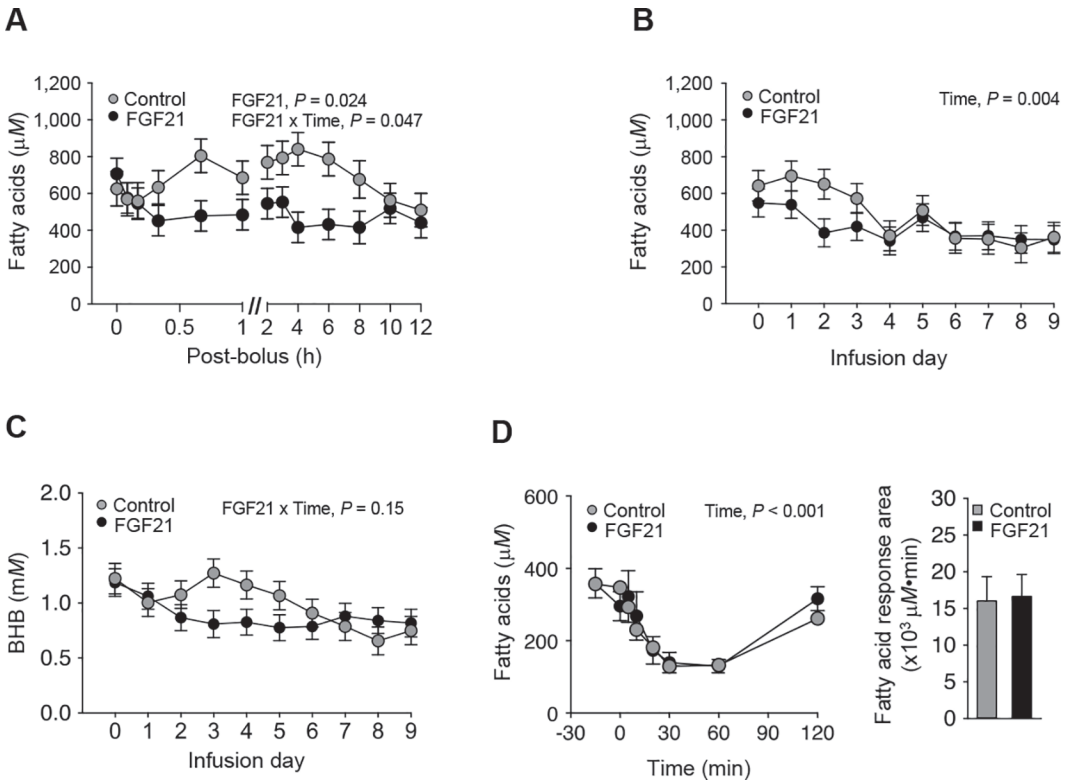

Figure 3. Effect of chronic administration of fibroblast growth factor-21 (FGF21) on lipid metabolism. An excipient (control) or human FGF21 solution was given i.v. as boluses on d 12.6 ± 2.2 of lactation (FGF21 dose = 3 mg/kg of BW) and as constant infusions (FGF21 dose = 6.3 mg/kg of metabolic BW per day) starting on d 14.6 ± 2.2. (A) Blood samples were collected over the 12-h period following the bolus, and plasma was analyzed for fatty acid concentration. Each point represents the LSM ± SE of 6 to 7 cows. The significant effects of FGF21 and FGF21 × Time are reported. (B) Blood samples were collected daily over the 9-d constant infusion, and plasma was analyzed for concentrations of fatty acids. Each point represents the LSM ± SE of 6 to 7 cows. The significant effect of Time is reported. (C) Blood samples were collected daily over the 9-d constant infusion, and plasma was analyzed for concentrations of BHB. Each point represents the LSM ± SE of 6 to 7 cows. The *P*-value for FGF21 × Time is reported. (D) A glucose tolerance test was administered on d 8 of the constant infusion. Blood samples were collected at set time points before and after administration of glucose (0.25 g/kg of BW). Plasma was analyzed for fatty acid concentrations, and the response area was calculated. Each point for fatty acid concentration and each bar for the fatty acid response area represent the LSM ± SE of 6 to 7 cows per treatment.

in an experimental design combining bolus and chronic administration directly into the vascular system. Intravenous bolus administration had the dual benefit of confirming human FGF21 bioactivity in cattle (Krumm et al., 2019) and allowing an estimation of its kinetics in the vascular compartment. This analysis yielded an estimated elimination $t_{1/2}$ of 3.2 h, which is in the same range as values obtained for native human FGF21 of 1.5 h in the mouse and 2 h in rhesus monkeys (Kharitonenkov et al., 2007; Xu et al., 2009b). We opted for i.v. rather than s.c. administration during the chronic phase to minimize loss related to incomplete transport into the vascular compartment and, more important, to limit proteolysis. Recent studies in mice and cynomolgus monkeys showed that human FGF21 is highly susceptible to cleavage of the last 10 AA and identified fibroblast activation protein as the responsible endopeptidase (Hecht et al., 2012; Hager et al., 2013). A similar situation could arise for bovine FGF21 because it also contains the C-terminal fibroblast activation protein proteolytic site. The loss of these AA ablates bioactivity, owing to the absolute requirement of the C-terminal region of FGF21 in recognizing its obligatory coreceptor β-klotho (Micanovic et al., 2009; Yie et al., 2012). Our data, however, suggested that this proteolytic cleavage is rather limited under our experimental conditions because plasma FGF21 concentrations detected by the intact assay, including in cows receiving human FGF21, did not differ significantly from those measured by the total assay.

Peripheral insulin resistance is regarded as one of the key adaptations of early lactation in dairy cows (Bell, 1995; Boisclair et al., 2006; De Koster and Opsomer, 2013). Insulin resistance creates a metabolic milieu favoring mammary glucose uptake, where it is used predominantly to synthesize lactose, the ultimate determi-

nant of milk volume (Bell and Bauman, 1997). On the other hand, FGF21 has been shown to reverse insulin resistance in rodents and primates suffering from type 2 diabetes or obesity (Xu et al., 2009a; Adams et al., 2013). Accordingly, one concern with chronic FGF21 therapy in dairy cows was reduced milk production arising as a consequence of its insulin-sensitizing effects. Chronic FGF21 therapy, however, did not alter milk production assessed as either milk yield or ECM yield. Absence of detrimental effects on milk production may relate to the inability of FGF21 to counter insulin resistance in energy-deficient, early-lactating dairy cows, as suggested by unaffected plasma glucose and insulin, glucose disposal during GTT, and adiponectin produc-

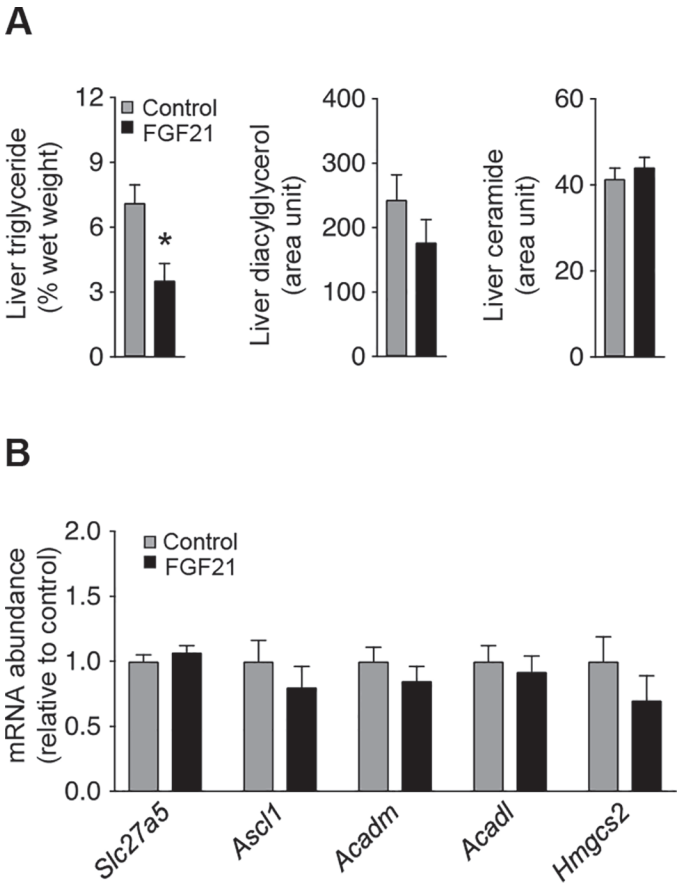

Figure 4. Effect of chronic administration of fibroblast growth factor-21 (FGF21) on liver lipids. Dairy cows received a constant i.v. infusion of an excipient (control) or human FGF21 (6.3 mg/kg of metabolic BW per day) starting on d 14.6 ± 2.2 of lactation and lasting for 9 consecutive days. (A) Biopsies of liver were obtained from dairy cows on d 9 of infusion and analyzed for total triglyceride, diacylglycerol, and ceramide. Each bar represents the LSM ± SE of the indicated variable (n = 6 cows). *$P < 0.036$. (B) Total RNA was isolated from the liver biopsies collected on d 9 of infusion and analyzed for the mRNA abundance of indicated genes. Expression of each gene is given relative to the control, with each bar representing the LSM ± SE of mRNA abundance for the indicated gene (n = 6 cows).

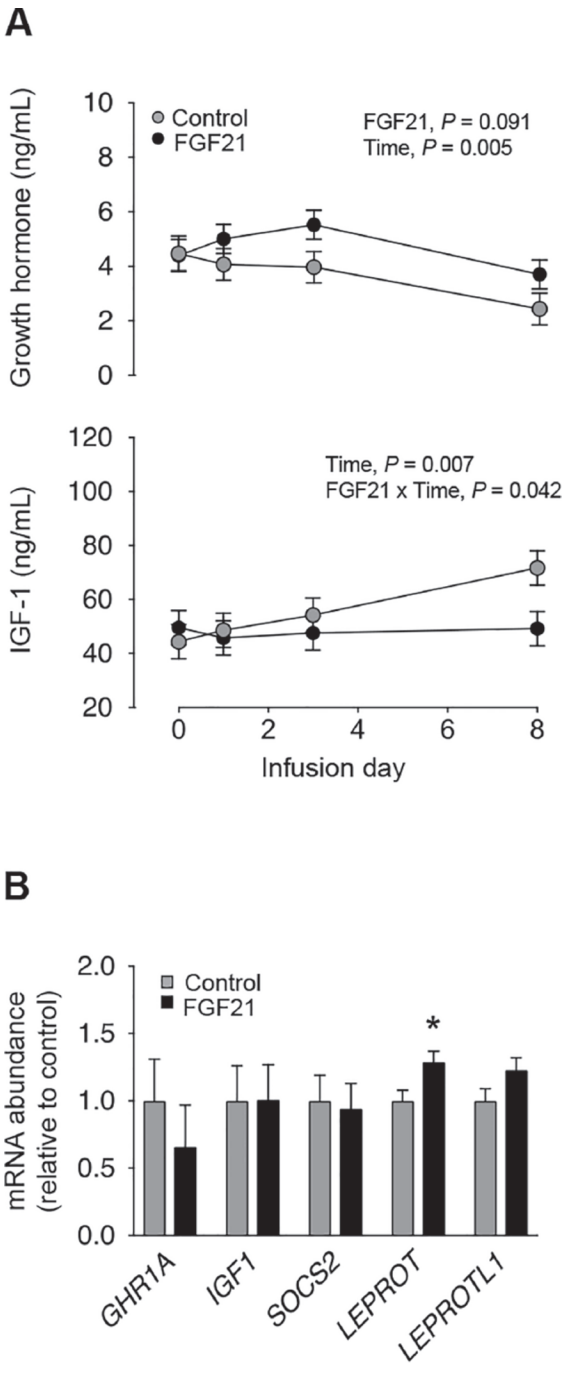

Figure 5. Effect of a chronic increase in plasma fibroblast growth factor-21 (FGF21) on growth hormone and IGF-1. Dairy cows received a constant i.v. infusion of an excipient (control) or human FGF21 (6.3 mg/kg of metabolic BW per day) starting on d 14.6 ± 2.2 of lactation and lasting for 9 consecutive days. (A) Blood samples were collected before and on d 1, 3, and 8 of the infusion, and plasma was analyzed for the concentration of growth hormone and IGF-1. Each point represents the LSM ± SE of 6 to 7 cows. The significant effects of FGF21, Time, and FGF21 × Time interaction are reported. (B) Total RNA was isolated from the liver biopsies collected on d 9 of infusion and analyzed for the mRNA abundance of indicated genes. Expression of each gene is given relative to the control, with each bar representing the LSM ± SE of mRNA abundance for the indicated gene (n = 6 cows). *$P = 0.02$.

tion (Krumm et al., 2019). Fibroblast growth factor-21 therapy has also been shown to depress voluntary feed intake in monkeys (Adams et al., 2013; Talukdar et al., 2016), raising the possibility that chronic treatment would exacerbate the negative energy balance of early lactation. This concern appears unwarranted because FGF21-treated cows did not experience reduced appetite, greater energy insufficiency, or lower BW and BCS at the end of treatment.

Liver accumulates triglyceride in early lactation because it takes up fatty acids in proportion to circulating levels, and this uptake outstrips its oxidative and secretory capacity (Drackley et al., 2001). Our data showed a 50% reduction in hepatic triglyceride levels in FGF21-treated cows. A similar effect in mice has been traced to reciprocal actions on pathways responsible for triglyceride synthesis and disposal. With respect to synthesis, FGF21 limits triglyceride accumulation in the mouse liver in part by inhibiting de novo fatty acid synthesis (Xu et al., 2009a; Fisher et al., 2014); this action cannot account for our results because this biosynthetic pathway is negligible in the ruminant liver (Bergen and Mersmann, 2005). More relevant to the dairy cow is the ability of FGF21 to reduce incorporation of adipose tissue–derived fatty acids into liver triglyceride. For example, mice lacking the transcription factor CREBH are unable to produce FGF21 upon fasting and experience increased lipolysis in adipose tissue, fatty acids in plasma, and triglyceride accumulation in liver; all 3 defects were corrected upon adenovirus-driven re-expression of FGF21 (Inagaki et al., 2007; Li et al., 2014; Park et al., 2016). Consistent with repressive effects of FGF21 on lipolysis, fasted FGF21 knockout (**KO**) mice have elevated plasma fatty acids and increased lipolytic activity in adipose tissue relative to their wild-type counterparts (Badman et al., 2009; Hotta et al., 2009). This antilipolytic effect was recapitulated in vitro in mouse and human primary adipocytes, suggesting that it is the result of direct FGF21 action on adipose tissue (Arner et al., 2008; Li et al., 2009). Our data provide some support for inhibition of lipolysis as a causal mechanism of reduced liver triglyceride as FGF21-treated cows experienced significantly lower plasma fatty acids after bolus administration and a numerical reduction during chronic administration.

Fibroblast growth factor-21 has also been shown to reduce liver lipid in rodents by stimulating fatty acid oxidation. Thus, FGF21 KO mice have more severe steatosis than their wild-type counterparts when fasted or consuming methionine- and choline-deficient diets, and this exacerbation is associated with depressed expression of lipid oxidative enzymes (e.g., *ASCL1*, *ACADM*, and *ADADL*), reduced rates of β-oxidation, and lower plasma BHB; remarkably, all of these effects were reversed when FGF21 KO mice received exogenous FGF21 therapy (Potthoff et al., 2009; Fisher et al., 2014; Tanaka et al., 2015). In contrast, we saw no effect of chronic FGF21 treatment on liver expression of key genes governing β-oxidation and, therefore, no evidence that increased β-oxidation accounts for reduced liver triglyceride in FGF21-treated cows. Finally, it is also possible that FGF21 stimulates VLDL secretion as mice with defective FGF21 expression accumulate less triglyceride in plasma when treated with the lipoprotein lipase inhibitor tyloxapol (Badman et al., 2009). Unlike oxidation, however, VLDL secretion is governed predominantly by posttranscriptional mechanisms (Choi and Ginsberg, 2011), and an assessment of the effect of FGF21 on VLDL secretion requires functional assays that were not possible in this work.

Early-lactating dairy cows experience a state of GH resistance as shown by depressed plasma IGF-1 despite elevated GH and by their inability to mount a robust GH-dependent increase in plasma IGF-1 (Rhoads et al., 2007). We and others have shown that one cause of GH resistance in liver is reduced GH receptor abundance as a consequence of decreased expression of the liver-specific GH receptor transcript *GHR1A* (Butler et al., 2003; Rhoads et al., 2007). Interestingly, FGF21-overexpressing mice exhibit hallmarks of GH resistance (elevated GH secretion and reduced plasma level of IGF-1) and suffer from significant deficits in linear and BW growth (Inagaki et al., 2008). Reciprocally, chronically undernourished FGF21 KO mice maintain elevated plasma IGF-1 and greater body and tibial growth relative to their wild-type counterparts, and this protection is obliterated by chronic FGF21 treatment (Kubicky et al., 2012). Fibroblast growth factor-21 triggers GH resistance via postreceptor mechanisms by inducing proteins that interfere with either trafficking of the GH receptor to the membrane (e.g., LEPROT and LEPROTL1) or its signaling (e.g., SOCS2; Inagaki et al., 2008; Wu et al., 2013). Our data reveal similar antagonism of GH action in early-lactating dairy cows, as FGF21-treated cows had lower plasma IGF-1 despite a tendency for increased plasma GH, and suggest induction of LEPROT as a mechanism in the liver.

In summary, we showed that FGF21 treatment reduces hepatic triglyceride accumulation in early-lactating dairy cows in the absence of negative effects on appetite, circulating BHB, and milk production. Future studies are needed to confirm that reduced influx of adipose tissue–derived fatty acids is the predominant mechanism accounting for reduced liver triglyceride and to ascertain whether this reflects a direct effect on adipose tissue.

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