Fibroblast growth factor 21 and exercise-induced hepatic mitochondrial adaptations

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Submitted 13 October 2015; accepted in final form 16 March 2016

The liver plays a vital role in maintaining euglycemia, in part through gluconeogenic processes during times of fasting or exercise. Hepatic mitochondria are instrumental in providing the ATP necessary to fuel this energy-costly process through the oxidation of fatty acids that are lipolyzed from hepatic and adipose stores (6). Exercise is a stimulus that challenges the liver to maintain euglycemia and hepatic mitochondria to produce the necessary ATP to maintain this endogenous glucose production. Evidence suggests that this exercise-induced challenge ultimately leads to greater hepatic mitochondrial content and function. Eight weeks of treadmill training has been shown to increase the activity of complexes I, IV, and V of the electron transport chain (41). In addition, daily voluntary wheel running led to greater complete palmitate oxidation, enzymatic activities of β-hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (markers of mitochondrial function and content, respectively), and the protein content of COX IV and cytochrome c in hyperphagic obese Otsuka Long-Evans Tokushima rats (34, 36). Recently, our laboratory has demonstrated that 4 wk of voluntary wheel running or treadmill training in healthy, lean Sprague-Dawley rats increases various indexes of hepatic mitochondrial function and content, including mitochondrial respiration, TCA cycle flux, pyruvate dehydrogenase activity, and citrate synthase activity (13). Despite the accumulating evidence that exercise training results in greater hepatic mitochondrial content and function, mechanisms mediating these changes have yet to be elucidated.

Fibroblast growth factor 21 (FGF21) is a novel endocrine factor that plays a key role in the regulation of carbohydrate and lipid metabolism (16). Exogenous administration of FGF21 or FGF21 analogs, as well as transgenic overexpression of FGF21, results in an elevation of hepatic metabolic processes such as β-oxidation, ketogenesis, TCA cycle flux, and oxygen consumption, which is accompanied by increased expression of gluconeogenic genes (Glucose 6-phosphatase, PEPCk), as well as those of cytochrome c, AMPKα1, CPT1α and CPT1β, and PGC-1α (5, 11, 32). While these changes are suggestive of improvements in mitochondrial function, the pharmacological levels of FGF21 achieved in these instances far exceed physiological fluctuations in FGF21, leaving many questions unanswered about the physiology of FGF21. In mice, the role of hepatic FGF21 in the physiology of fasting has been most extensively characterized and has been shown to be involved in the regulation of mitochondrial fatty acid oxidation and ketone body production (2, 27, 32). More recently, elevations in FGF21 have been reported with exercise and it has been proposed that FGF21 may mediate some of the metabolic benefits of exercise (8, 21, 22). However, a positive correlation between exercise and FGF21 is not always reported and it appears that the duration and intensity of the exercise along with the time point at which blood samples are taken may all influence FGF21 levels, making an interpretation of these data more difficult (25, 31, 39). Therefore, the purpose of the present investigation was to directly determine the role of FGF21 in exercise-induced...
hepatic mitochondrial adaptations in FGF21 knockout mice. It was hypothesized that FGF21 knockout mice would not display the same exercise-induced adaptations in hepatic metabolic processes and gene transcription that would occur in wild-type (WT) mice with normal FGF21 signaling. Findings from this research may aid in identifying specific mechanisms responsible for hepatic mitochondrial adaptations induced by exercise.

METHODS

Animal protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia and Harry S Truman Memorial Veterans Affairs Hospital. FGF21 knockout mice on a C57BL/6 background (FGF21-KO) and age-matched, but not littermate C57BL/6 WT mice controls were bred by Taconic Biosciences (Hudson, NY) and kindly provided by Eli Lilly (Indianapolis, IN). Male FGF21-KO and WT mice (11–12 wk of age) were provided with a running wheel to promote voluntary wheel running, the chosen mode of exercise, for 8 wk and were designated as FGF21-KO-EX (n = 14) and WT-EX (n = 15). Separate groups of FGF21-KO (n = 9) and WT (n = 10) mice remained sedentary without access to running wheels and were designated as FGF21-KO-SED and WT-SED. All mice were individually housed during the intervention in temperature-controlled animal quarters (21°C) with a 0600–1800 light and 1800-0600 dark cycle. All groups were provided standard rodent chow (Formulab 5008; Purina Mills, Brentwood, MO) for ad libitum feeding in new cages at the beginning of each week. Running wheel revolutions were monitored and counted continuously through the 8-wk intervention by use of a Sigma BC 509 bike computer (St. Charles, IL). Running distance was obtained every day between 0800 and 1000. Body mass and food consumption were measured on the same day each week throughout the study. To calculate feeding efficiency, a measurement that provides an index of energy balance, the change in body weight was divided by the amount of food consumed [Δ body weight (g)/g intake] during defined periods.

After the 8-wk intervention, running wheels were locked for 24 h and all mice were then fasted for 5 h, anesthetized with pentobarbital sodium (100 mg/kg), and then exsanguinated by removal of the heart. Retroperitoneal, epididymal, and inguinal adipose tissue fat pads were excised and weights were recorded.

Body composition. Fat mass, lean mass, and percentages of body fat and lean mass were measured with an EchoMRI 4in1-1100 analyzer (EchoMRI; Houston, TX).

Tissue homogenization and mitochondrial isolation procedures. Livers were quickly excised from anesthetized mice and either flash frozen in liquid nitrogen or placed in ice-cold isolation buffer (in mM: 220 mannitol, 70 sucrose, 10 Tris-base, 1 EDTA; pH 7.4). Hepatic mitochondria were prepared as previously described (28, 38).

Palmitate oxidation. Oxidation of [1-14C]palmitate (American Radiochemicals; St. Louis, MO) was measured in whole liver homogenate and fresh isolated hepatic mitochondria preparations. The collection and measurement of 14CO2 palmitate oxidation allowed for the estimation of complete fatty acid oxidation and was conducted as previously described (34).

Citrate synthase and β-HAD activity. Hepatic citrate synthase and β-HAD activity were measured as previously described by Srere (40) and Bass et al. (3), respectively, as well as previously described by our laboratory (34).

Intrahepatic lipid content and liver morphology. Liver tissue was formalin fixed and paraffin embedded. Hematoxylin and eosin (H&E) stains were done to examine liver morphology. Intrahepatic triacylglycerol content was determined by a biochemical assay as previously described (34).

Hepatic glycogen content. Glycogen content in liver tissue was measured as previously described by Aschenbach et al. (1) and by our laboratory (34).

Gene expression. Phosphoepinephrine carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), peroxisome proliferator-activated receptor-α (PPARα), peroxisome proliferator-activated receptor-γ co-activator 1-α (PGC-1α), carnitine palmitoyltransferase 1 (CPT1α), fibroblast growth factor receptor substrate 2 (FRS2), β-Klotho, and mitochondrial transcription factor A (TFAM) mRNA expression were quantified as previously described (35). Briefly, to examine PEPCK and G6Pase, the primer dilution mix [nuclease-free water, and both forward and reverse primers (Sigma), Fast SYBR Green Master Mix kit (ABI)] and cDNA sample (50 ng) were loaded to a 96-well microplate and placed into the ABI 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, CA) for polymerization. FGF21 gene expression was assessed by loading TaqMan Master Mix (ABI), FGF21 primers and probe (Applied Biosystems), and cDNA samples (50 ng) into a 96-well microplate, which was placed into the ABI 7500 Fast Sequence Detection System (Applied Biosystems) for polymerization. Once polymerization was completed, results were quantified by the DDΔΔCt technique relative to the cyclophilin-B (cycB) housekeeping gene.

Serum measurements. Serum glucose, aspartate aminotransferases, alanine aminotransferases, and lipids were all determined on the Cobas c311 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Serum ketones were measured with a commercially available β-hydroxybutyrate Liquicolor kit (Stanbio, Boerne, TX). Serum total FGF21 (mouse/rat FGF21 kit Quantikine ELISA; R&D Systems, Minneapolis, MN), insulin (ultrasensitive mouse insulin ELISA kit; Crystal Chem, Downers Grove, IL), and adiponectin (mouse adiponectin ELISA kit; BioVender, Asheville, NC) were measured by using commercially available assays and following manufacturer-provided protocols.

Nuclear extraction. Nuclear and cytoplasmic protein fractions from liver were isolated using by a commercially available NE-PER nuclear and cytoplasmatic extraction reagents kit (Thermo Scientific, Rockford, IL).

Western blotting. Western blot analyses were performed to determine protein content of the following: oxidative phosphorylation (OXPHOS) electron transport chain complexes I through V (Mito-Profile Total OXPHOS Rodent WB Antibody Cocktail; Abcam, Cambridge, MA), PGC-1α (EMD Millipore, Billerica, MA), mitochondrial transcription factor A (TFAM; Santa Cruz Biotechnology, Dallas, TX), PEPCK (Santa Cruz Biotechnology), G6Pase (Santa Cruz Biotechnology), PPARα (Santa Cruz Biotechnology), fibroblast growth factor receptor 2 (FGFR2; Abcam), FRS2 (Abcam) and phospho-FRS2 (Cell Signaling, Danvers, MA), β-Klotho (Santa Cruz Biotechnology), pyruvate carboxylase (PC; Abcam), cAMP-responsive element-binding protein (CREB) and phospho-CREB(Ser133) (Cell Signaling), AMP-activated protein kinase (AMPK) and phospho-AMPK(Thr 172) (Cell Signaling), and acetyl-CoA carboxylase (ACC) and phospho-ACC (Cell Signaling).

Phosphorylation status (with phosphospecific antibodies) was calculated from the density of the phosphoprotein band divided by density of the total protein with use of the appropriate antibody (33, 34). Membranes stained with 0.1% amido black (Sigma) were quantified to control for differences in protein loading or transfer of band densities as previously described (34). Hepatic FGF21 protein content was assessed with a commercially available mouse/rat FGF21 kit Quantikine ELISA (R&D Systems).

Statistics. Each outcome measure was examined in 9 to 15 animals per group. Main effects of exercise (SED vs. EX) and genotype (WT vs. FGF21-KO) were examined through a two-way analysis of variance (IBM SPSS, version 20.0, SPSS, Chicago, IL), and significant main effects (0.05) were followed with Fisher least significant difference post hoc comparisons. Values are reported as means ± standard deviation post hoc comparisons. Values are reported as means ± standard deviation post hoc comparisons.
errors (SE), and a P value of ≤0.05 denotes a statistically significant difference.

RESULTS

Animal characteristics. WT-EX and FGF21-KO-EX groups ran, on average, 7.8 and 7 km (respectively) each night with no significant difference in average weekly running distance between the two groups (P ≥ 0.05; Table 1). Heart mass to-body mass ratio was significantly higher in the EX groups (WT-EX and FGF21-KO-EX) compared with their SED counterparts (WT-SED and FGF21-KO-SED) (P ≤ 0.05; Table 1), indicating an adequate stimulus for training adaptations.

FGF21-KO mice had greater body weight and adiposity than WT (P < 0.001), while EX lowered these two variables in both genotypes (P < 0.001; Table 1; Fig. 1). Furthermore, lean mass was elevated in the FGF21-KO mice (P < 0.05; Table 1). In examining percentages of body fat and lean mass (P < 0.05), an absence of FGF21 resulted in a greater percentage of body fat and a reduction in percentage of lean mass compared with WT mice. Additionally, EX lowered the percentage of body fat while increasing the percentage of lean mass compared with SED animals in each genotype (P < 0.05; Table 1). These observed increases in body weight and adiposity are, in part, due to the greater food consumption in the FGF21-KO mice (P < 0.05). EX also led to greater food intake (P < 0.05; Table 1). Despite this greater food consumption, 8 wk of EX still lowered body weight as is reflected in significant reductions in feed efficiency (P < 0.05; Table 1). Furthermore, a lack of FGF21 and/or exercise resulted in a significantly greater liver mass to body mass ratio (P < 0.05; Table 1). In addition to lowering body weight and adiposity, EX also reduced hepatic triglycerides in both genotypes (P < 0.05; Fig. 2). Representative H&E images of the liver indicate greater lipid vacuolization in the FGF21-KO-SED mice compared with WT mice, which was normalized with 8 wk of EX (Fig. 2).

Eight weeks of EX did not alter circulating FGF21 concentrations in the WT mice. However, FGF21 deficiency caused elevations in 5-h fasted serum cholesterol, insulin, and glucose concentrations compared with WT mice (P < 0.05); EX effectively lowered serum triglycerides, free fatty acids, insulin, and adiponectin in WT and FGF21-KO mice (P < 0.05, Table 2). Furthermore, FGF21-KO mice displayed evidence of suppressed ketogenesis, with ~40% lower serum ketones (β-hydroxybutyrate) compared with WT mice (P < 0.05; Table 2).

Markers of hepatic mitochondrial function. A major finding in the present investigation is that FGF21 deficiency reduced complete disposal of palmitate oxidation to CO₂ in isolated liver mitochondria, yet this effect was completely rescued with daily EX (P < 0.05; Fig. 3A). Examining complete [1-14C]palmitate oxidation in whole homogenates again that EX increases fatty acid oxidation and this effect is likely driven...
by the significant increase in fatty acid oxidation in the FGF21-KO-EX mice compared with the FGF21-KO-SED group \((P < 0.05; \text{Fig. 3B})\). Moreover, lack of FGF21 lowered the activity of \(\beta\)-HAD \((P = 0.001; \text{Fig. 3C})\), the rate-limiting enzyme in \(\beta\)-oxidation, a reduction that was not reversed with EX. Interestingly, EX suppressed hepatic citrate synthase activity in both genotypes \((P < 0.05; \text{Fig. 3D})\).

Additional analyses were performed on liver tissue to examine several key regulators of fat oxidation in an attempt to help explain the observed reduction of complete palmitate oxidation in liver TGs.

**Table 2. Serum measures**

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT-SED</th>
<th>WT-EX</th>
<th>FGF21KO-SED</th>
<th>FGF21KO-EX</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF21, pg/ml</td>
<td>892.50 ± 199.63</td>
<td>858.60 ± 130.97</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.23 ± 0.10</td>
<td>0.82 ± 0.08†</td>
<td>2.61 ± 0.29*</td>
<td>1.95 ± 0.23†*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>280.20 ± 10.75</td>
<td>280.53 ± 15.77</td>
<td>332.22 ± 21.89*</td>
<td>350.84 ± 13.21*</td>
</tr>
<tr>
<td>(\beta)-Hydroxybutyrate, (\mu)mol/l</td>
<td>41.64 ± 4.29‡</td>
<td>28.04 ± 2.69†</td>
<td>27.12 ± 2.38*</td>
<td>24.04 ± 2.13‡*</td>
</tr>
<tr>
<td>Adiponectin, (\mu)g/ml</td>
<td>21.38 ± 1.05</td>
<td>18.10 ± 0.64†</td>
<td>20.17 ± 0.92</td>
<td>17.85 ± 0.49†</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>61.06 ± 4.69</td>
<td>47.33 ± 2.10†</td>
<td>64.44 ± 3.91</td>
<td>57.89 ± 2.53†</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>95.37 ± 4.96</td>
<td>91.42 ± 1.90</td>
<td>111.51 ± 4.28*</td>
<td>99.10 ± 4.28*</td>
</tr>
<tr>
<td>FFA, (\mu)M</td>
<td>769.44 ± 53.55</td>
<td>598.89 ± 32.73‡</td>
<td>813.11 ± 26.14</td>
<td>680.22 ± 35.05‡</td>
</tr>
</tbody>
</table>

Values are means ± SE \((n = 9–15)\). *Significant genotype main effect \((P < 0.05)\); †significant exercise main effect \((P < 0.05)\); ‡significant differences vs. WT-EX and FGF21KO-SED \((P < 0.05)\). BDL, below detectable limits; TG, triglyceride; FFA, free fatty acid.
the FGF21-KO-SED group and how EX rescued the deficit. CPT-1α was examined because of its role in fatty acid entry into the mitochondria, and it was observed that EX upregulated (P < 0.05; Fig. 3E) this important gene involved in fatty acid oxidation in both WT and FGF21-KO mice. The ratio of phosphorylated AMPK to total AMPK hepatic protein content was then measured because of the ability of AMPK to act as an energy sensor and ability to upregulate fatty acid oxidation in the liver during times of energy deprivation. Although AMPK and phosphorylation status of AMPK were not different among groups (Fig. 3F), ACC phosphorylation status (phosphorylated ACC/total ACC) at Ser-79 [inhibitory site (17) that is known to be phosphorylated by AMPK] was increased with EX in both WT and FGF21-KO mice (P < 0.05; Fig. 3G), albeit both phosphorylated ACC and total ACC protein content was lowered by EX (P < 0.05; representative images shown in Fig. 3H).
Proteins involved in mitochondrial biogenesis. We assessed PGC-1α, a potent coactivator of transcriptional processes that plays a vital role in multiple metabolic processes, including fatty acid oxidation and mitochondrial biogenesis. Neither the ablation of FGF21 nor 8 wk of EX altered PGC-1α protein content or mRNA expression (Fig. 4, A and B, respectively). Transcriptional (active) PGC-1α resides in the nucleus where it can actively coactivate transcription factors that bind to DNA. It was found that FGF21-KO mice had significant reductions in the nuclear content of PGC-1α (P < 0.05; Fig. 4C), which was not rescued by exercise. The protein content of PPARα, a transcription factor involved in the transcription of fatty acid oxidation related genes and FGF21, was elevated in the FGF21-KO mice compared with WT animals (P < 0.05, Fig. 4D). However, PPARα mRNA expression was not affected by EX or genotype (Fig. 4E).

To examine the effects of the observed reduction in the nuclear content of PGC-1α on mitochondrial biogenesis, we measured hepatic TFAM, an important transcription factor involved in mitochondrial biogenesis, and OXPHOS protein content. Despite reduction in the nuclear content of PGC-1α, FGF21-KO mice had greater protein content and gene expression of TFAM (P < 0.05; Fig. 4, F and G, respectively). Interestingly, TFAM mRNA expression was lowered with EX in FGF21-KO mice. When examining the complexes in OXPHOS, we found that EX reduced complex IV protein content in both genotypes (P < 0.05; Fig. 4H).

Gluconeogenesis. Mechanisms involved in gluconeogenesis were analyzed to determine how they were influenced by EX and/or a lack of FGF21. The hepatic protein content of activated CREB (phosphorylated CREB/total CREB), a transcription factor responsible for transcribing gluconeogenic genes, was found to not be different among groups (Fig. 5A). However, mRNA expression of PEPCK, another important enzyme involved in gluconeogenesis, was significantly increased in the WT-EX mice (P ≤ 0.001), but not in FGF21-KO-EX mice (Fig. 5B). FGF21-KO mice also had lower PEPCK protein content compared with WT mice (P ≤ 0.001), which was not affected by EX (Fig. 5C). Unlike PEPCK, G6Pase mRNA expression was not affected by EX or ablating FGF21 (Fig. 5D); however, EX did lower the protein content of both G6Pase (P < 0.01; Fig. 5E) and PC (P ≤ 0.001; Fig. 5F).

Hepatic glycogen is another contributor to endogenous glucose production and may affect the need for gluconeogenesis, as well as the protein content and gene expression of important enzymes involved in gluconeogenesis and/or vice versa. In the present report, hepatic glycogen content was elevated in FGF21-KO mice (P < 0.05; Fig. 5G).

FGF21 signaling mediators. We have previously reported that exercise increased and/or rescued FGF21 signaling mediators in OLETF rats when exercise was used to prevent obesity (12). In the present investigation, the effects of exercise and/or a lack of FGF21 on FGF21 signaling mediators was assessed. To confirm the deletion of FGF21, hepatic FGF21 mRNA expression and protein content were assessed. FGF21 was not detected in FGF21-KO mice, and neither gene expression nor protein content differed between the WT-SED and WT-EX mice (Fig. 6, A and B, respectively). Furthermore, neither protein content nor gene expression of β-Klotho or FGFR2 was affected by EX or a lack of FGF21 (Fig. 6). FRS2 protein content was also not affected by either treatment; however, FRS2 gene expression was increased by 8 wk of EX in both genotypes (P < 0.05; Fig. 6).

DISCUSSION

Exercise is a known stimulus for increasing hepatic mitochondrial function (13), yet the mechanisms responsible for these adaptations remain to be elucidated. FGF21, a powerful metabolic regulator known to be induced by fasting (4, 15), is one potential mediator of exercise-induced adaptations. The present investigation examined, for the first time, the role of FGF21 in improvements in mitochondrial function that occur in response to exercise. Here we report novel findings suggesting that FGF21 does not appear necessary for exercise-induced hepatic mitochondrial adaptations and that impairments in hepatic fatty acid oxidation induced by FGF21 deficiency can be partially rescued by daily wheel running exercise.

In the present study, lack of FGF21 resulted in low-fat chow-fed mice developing greater body weight, adiposity, hyperinsulinemia, hyperglycemia, and dyslipidemia in sedentary mice. We also demonstrate that 8 wk of voluntary wheel exercise can largely normalize most of these abnormalities. The oxidation of fatty acids is the primary means of energy production in the liver to support other processes such as endogenous glucose production (gluconeogenesis). In the present investigation, as expected, a lack of FGF21 resulted in diminished palmitate oxidation in sedentary mice. Importantly, 8 wk of EX normalized the effects of FGF21 deficiency on hepatic fatty acid oxidation. We have previously found that 6, 16, and 36 wk of daily voluntary wheel running prevents obesity and enhances (24, 34, 36) fatty acid oxidation in hyperphagic OLETF rats and that 4 wk of daily voluntary wheel running induced a 40% increase in complete palmitate oxidation in healthy lean Sprague-Dawley rats compared with sedentary rats (13). Here, 8 wk of daily voluntary wheel running did not increase complete palmitate oxidation in the healthy lean wild-type mice. The reason for the discrepancy is unknown, but it is possible that adaptations in hepatic fat oxidation with exercise take longer in mice compared with rats (13, 24).

Our findings that a deficit of hepatic fatty acid oxidation occurs in the absence of FGF21 agrees with previous reports (10, 32), and it is well documented that FGF21 also regulates whole body fat oxidation and energy expenditure (7, 29, 45). These changes in fat oxidation and energy expenditure due to a lack of FGF21 most likely played a major role in the elevated body weight found in FGF21 KO mice.

FGF21 increases hepatic fatty acid oxidation (32), as well as transcription of genes important to this process (26). Acute FGF21 administration leads to increases in hepatic PGC-1α gene expression (11, 32), whereas administration of FGF21 for 8 days results in the increased gene expression of CPT1α, CPT1β, and AMPKα1. Moreover, knocking down FGF21 gene expression leads to a failure to upregulate genes involved in hepatic β-oxidation (medium, long, and very long chain acyl-CoA dehydrogenase), and CPT1α in response to a ketogenic diet (2). Here we report that a lack of FGF21 resulted in reduced hepatic palmitate oxidation in sedentary mice, which was normalized with daily wheel running exercise in the absence of changes in PGC-1α or PPARα protein content and gene expression. Although PGC-1α transcriptional activity was not directly assessed, nuclear protein content of PGC-1α...
Fig. 4. Effects of exercise and/or absence of FGF21 on PGC-1α protein content (A), PGC-1α mRNA expression to WT-SED (B), PGC-1α protein content found in the nucleus (C), PPARα protein content (D), PPARα mRNA expression to WT-SED (E), TFAM protein content (F), TFAM mRNA expression to WT-SED (G), and oxidative phosphorylation (H). Representative Western blots shown in I. Representative images do not reflect the order in which they appear in the graphs. WT, wild-type; FGF21-KO, FGF21 knockout; SED, sedentary; EX, exercise. Values are means ± SE (n = 9–15). #Significant genotype main effect, P ≤ 0.05; †significant differences vs. WT-SED and FGF21KO-EX, P ≤ 0.05. A.U., arbitrary units.
was found to be lower in the FGF21-KO groups, and 8 wk of EX failed to change the nuclear content of this important cotranscription factor. It is entirely possible that exercise upregulated other transcription factors, and/or changes in PPAR or PGC-1 content or location were not necessary to accommodate the necessary increases in genes associated with fatty acid oxidation. PGC-1 cotranscribes CPT-1 (37), and an increase in CPT-1 mRNA expression was observed in the FGF21-KO group with EX despite a reduction in nuclear PGC-1.

The upregulation in CPT-1 may also explain the EX-induced increase in fatty acid oxidation, since entry into the mitochondria through CPT-1 is the rate-limiting step of β-oxidation (9, 20, 30). This step in the process can be inhibited by ACC activity, the enzyme responsible for the rate-limiting step of fatty acid synthesis and the production of malonyl-CoA. Malonyl-
CoA has an inhibitory effect on CPT-1α and can therefore limit fat oxidation (9, 30). ACC is deactivated upon phosphorylation on ser79 by AMPK (9, 17). In the present study, EX increased hepatic ACC ser79 phosphorylation status (increased ratio of phosphorylated ACC to total ACC) in both FGF21-KO and WT mice, which is indicative of increased AMPK activity. Taken together, these data suggest that exercise, at least in part, rescued fatty acid oxidation in FGF21-KO mice by increasing AMPK activity and promoting increased fatty acid entry into the mitochondria through CPT-1α.

Not only is PGC-1α important for hepatic fatty acid oxidation, it is also vital to mitochondrial biogenesis. PGC-1α aids...
in the transcription of nuclear respiratory factor-1 (NRF-1), which in turn is responsible for the transcription of TFAM, an important transcription factor for mitochondrial biogenesis (44). TFAM, transcribed from nuclear DNA, is then able to translocate to mitochondrial DNA and transcribe proteins from the mitochondrial genome including subunits of electron transport chain complexes (43, 44). Despite a reduction in hepatic nuclear PGC-1α protein content, FGF21-KO mice had greater TFAM protein content and gene expression. However, this elevation in TFAM in the FGF21-KO mice did not appear to affect the protein content of electron transport chain complexes. Surprisingly, hepatic complex IV protein content was actually reduced in EX groups; the reason for this reduction is unknown. The protein content of these electron transport chain complexes are considered markers of mitochondrial content (23). Citrate synthase activity, another marker of mitochondrial content (23), was also reduced in response to voluntary wheel running. These findings contrast those of a previous wheel running study from our group using rats (36). Discrepancies in these findings may be due to a larger liver weight-to-body weight ratio in voluntary wheel running mice compared with sedentary mice. Perhaps a larger liver-to-body weight ratio reduces the gluconeogenic burden in these mice and increased mitochondrial density is not needed, but rather the quality of the mitochondria is increased. This is strictly speculation and these findings warrant future, more in-depth studies.

The transcription factor CREB and cotranscription factor PGC-1α are responsible for upregulating hepatic gluconeogenic genes. Previous studies have shown that this upregulation of gene expression is stimulated both by FGF21 signaling as well as exercise (13, 14, 32). Although exercise and the deletion of FGF21 did not influence the protein content of PGC-1α or phosphorylated CREB in the present report, the absence of FGF21 resulted in a blunting of PEPPCK content in both the sedentary and voluntary wheel running groups. Furthermore, 8 wk of voluntary wheel running upregulated the hepatic gene expression of PEPPCK in WT mice yet was unable to do so in the absence of FGF21. Large decreases in PEPPCK protein content have been shown to lead to small reductions in gluconeogenesis (6), and it is not known whether the reductions in PEPPCK protein content in the FGF21-KO groups were sufficient to lower gluconeogenesis in these mice. However, if a decrement in gluconeogenesis did occur in the FGF21-KO mice, then it is possible that the greater glycogen content in these animals was a compensatory mechanism to help maintain endogenous glucose production. Using Sprague-Dawley rats, our laboratory previously found that PEPPCK mRNA expression and the protein content of phosphorylated CREB increased in response to voluntary wheel running in a fasted condition, while hepatic G6Pase expression remained unchanged (13). Similarly, in the present report, whereas 8 wk of EX increased PEPPCK expression, the protein content of the gluconeogenic enzymes G6Pase and PC were decreased. Taken together, these data suggest that not all gluconeogenic enzymes respond to an exercise stimulus in the same manner. Similar findings have been previously reported (18). Importantly, these data also suggest that wheel running exercise does not have the same ability to upregulate hepatic PEPPCK in the absence of FGF21.

We have previously demonstrated that voluntary wheel running for 36 wk not only prevents obesity in hyperphagic OLETF rats but also increases or rescues FGF21 signaling mediators (β-Klotho, FGR2, and FRS2) while at the same time lowering circulating FGF-21 levels (12). In the present study, 8 wk of voluntary wheel running exercise increased hepatic FRS2 mRNA expression in both genotypes but did not affect hepatic gene expression or protein content of FGF21 or any of the other FGF21 signaling mediators assessed. Additionally, exercise did not alter circulating FGF21 levels in the WT mice. The literature regarding exercise-induced changes in FGF21 is inconsistent. Although some studies have found increases in FGF21 with a single exercise bout (4, 21) and that the liver contributes to the release of FGF21 during exercise (19), others have reported changes after weeks of training but no changes following acute exercise (8). Moreover, consistent with our observations in obese rats (12), circulating FGF-21 levels are reduced following exercise training in overweight and obese individuals (39, 46) and in the elderly following 5 wk of cycle ergometer (42). The discrepancies in the literature may be due to the exercise conditions studied, the timing of blood collection in relation to the last exercise bout, and/or a number of other variables known to influence FGF21 levels both acutely and chronically including diet composition, fasting duration, and the health/age/sex/species of the subject/animal tested.

The use of FGF21-KO mice provided a means to directly determine the extent to which FGF21 contributes to exercise-associated improvements in liver metabolism. Collectively, our data do not support a strong role for exercise-induced enhancement of FGF21 signaling in mediating exercise-induced increases in hepatic fatty acid oxidation. However, given the pleotropic nature of FGF21, it is possible that it is important for exercise-associated benefits in other tissues, and additional investigations are warranted to examine this more thoroughly.

In conclusion, data from the present investigation indicate that FGF21 deficiency results in greater body weight, adiposity, dyslipidemia, and insulin resistance in mice fed a low-fat chow diet. In addition, FGF21 deficiency led to reductions in markers of hepatic mitochondrial function and biogenesis. However, our findings suggest that FGF21 may not be necessary for exercise-induced systemic and hepatic mitochondrial adaptations, since 8 wk of daily wheel running exercise effectively corrected many, but not all, of these abnormalities. Importantly, daily exercise normalized impairments in hepatic fatty acid oxidation induced by FGF21 deficiency. Collectively, these data further support the metabolic importance of both intact FGF21 signaling and daily exercise on liver health and also highlight some of the independent effects of each on these metabolic processes.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Radhey Naik, Tasnim Farha Haq, and Kayla Kanosky. This work was supported with resources and the use of facilities at the Harry S Truman Memorial Veterans Hospital in Columbia, MO.

GRANTS

This research was supported by an ACSM Foundation Research Grant from the American College of Sports Medicine Foundation (J. A. Fletcher), as well as National Institutes of Health Grants T32 AR 048523-07 (J. A. Fletcher and E. M. Morris) and DK-088940 (J. P. Thyfault) and Veterans Affairs Grants Merit (I01BX002567-01, J. P. Thyfault) and VHA-CD22 4K2BX001299 (R. S. Rector).
DISCLOSURES
A. Butterfield and J. W. Perfield II are paid employees of Eli Lilly and Company and may own company stock or possess stock options. The remaining authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

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