Anti-inflammatory effects of exercise training in adipose tissue do not require FGF21

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Abstract

Exercise enhances insulin sensitivity; it also improves adipocyte metabolism and reduces adipose tissue inflammation through poorly defined mechanisms. Fibroblast growth factor 21 (FGF21) is a pleiotropic hormone-like protein whose insulin-sensitizing properties are predominantly mediated via receptor signaling in adipose tissue (AT). Recently, FGF21 has also been demonstrated to have anti-inflammatory properties. Meanwhile, an association between exercise and increased circulating FGF21 levels has been reported in some, but not all studies. Thus, the role that FGF21 plays in mediating the positive metabolic effects of exercise in AT are unclear. In this study, FGF21-knockout (KO) mice were used to directly assess the role of FGF21 in mediating the metabolic and anti-inflammatory effects of exercise on white AT (WAT) and brown AT (BAT). Male FGF21KO and wild-type mice were provided running wheels or remained sedentary for 8 weeks (n=9–15/group) and compared for adiposity, insulin sensitivity (i.e., HOMA-IR, Adipo-IR) and AT inflammation and metabolic function (e.g., mitochondrial enzyme activity, subunit content). Adiposity and Adipo-IR were increased in FGF21KO mice and decreased by EX. The BAT of FGF21KO animals had reduced mitochondrial content and decreased relative mass, both normalized by EX. WAT and BAT inflammation was elevated in FGF21KO mice, reduced in both genotypes by EX. EX increased WAT Pgc1alpha gene expression, citrate synthase activity, COX I content and total AMPK content in WT but not FGF21KO mice. Collectively, these findings reveal a previously unappreciated anti-inflammatory role for FGF21 in WAT and BAT, but do not support that FGF21 is necessary for EX-mediated anti-inflammatory effects.

Key Words
- exercise
- inflammation
- FGF21
- adipose tissue
Introduction

Two major types of adipose tissue (AT) exist: white (WAT), primarily serving as the body’s major energy storage site and brown (BAT), which predominately converts energy into heat through uncoupled respiration. AT is an active endocrine organ that is profoundly affected by exercise. Exercise lessens WAT inflammation and improves systemic metabolic health, even in the settings of obesity (Vieira et al. 2009a, Welly et al. 2016), although the mechanisms are not fully understood. Less is known about the effect of exercise on BAT inflammation. In this regard, we recently showed that exercise training also reduces obesity-induced BAT inflammation in rodents (Wainright et al. 2015, Welly et al. 2016). In addition, evidence suggests that exercise increases WAT mitochondrial content and function (Stallknecht et al. 1991, Lee et al. 2014, Wainright et al. 2015, Stanford & Goodyear 2016), which is associated with metabolically healthier WAT. Accordingly, the exercise-induced changes in WAT (and possibly BAT) mitochondria may contribute to some of the metabolic benefits of exercise (Stanford et al. 2015). Yet, the specific mechanisms by which exercise reduces inflammation and induces mitochondrial adaptations are not completely understood.

Fibroblast growth factor 21 (FGF21), a pleiotropic endocrine hormone produced by several tissues, plays an important role in systemic glucose and lipid metabolism (Kharitonenkov et al. 2005, Zhang et al. 2008, Iglesias et al. 2012, Kim & Lee 2014, Samms et al. 2015). FGF21 enhances adipocyte glucose uptake and improves insulin resistance and diabetes (Kharitonenkov et al. 2005, Kharitonenkov & Shanafelt 2008, Kim & Lee 2014, Samms et al. 2015). Importantly, it exerts the majority of its systemic beneficial effects via its actions in AT. Indeed, in the absence of intact receptor signaling in AT, the majority of FGF21’s metabolic effects are nullified (Adams et al. 2012). FGF21 reduces WAT mass and enhances energy expenditure by activating BAT. Similar benefits have been shown with exercise training (Kim et al. 2013, Kim & Lee 2014, Ji et al. 2015), which has been shown to increase FGF21 secretion in some human and rodent studies (Kim et al. 2013, Loyd et al. 2016, Tanimura et al. 2016). Recently, anti-inflammatory properties of FGF21 have been reported (Feingold et al. 2012, Singhal et al. 2016, Yu et al. 2016); however, whether exercise-induced anti-inflammatory actions in AT are mediated via FGF21 has not been addressed.

The purpose of the current investigation was to determine the role that FGF21 plays in exercise training-mediated metabolic adaptations in WAT and BAT. We and others have consistently observed robust anti-inflammatory effects of exercise in AT (Vieira et al. 2009a,b, Castellani et al. 2014, Welly et al. 2016), but the mechanisms underlying this observation are not well understood. Here, we tested the hypothesis that lack of FGF21 leads to a dysfunctional AT phenotype. Further, we determined if FGF21 is necessary for the anti-inflammatory effects of exercise training in WAT and BAT using a mouse model of FGF21 ablation.

Materials and 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 VA Hospital. Male FGF21-knockout mice on a C57BL/6NTac background (FGF21KO) and age matched, but not littermate, C57BL/6NTac WT mice controls were bred by Taconic Biosciences (Hudson, NY) and kindly provided by Eli Lilly (Indianapolis, IN). FGF21 KO mice were generated by backcrossing with C57BL6 mice for >15 generations. Prior experiments and experience with this model determined that WT littermates and age-matched C57BL6 animals were phenotypically indistinguishable. FGF21KO and WT mice (11–12 weeks of age) were provided a running wheel for 8 weeks and designated as FGF21KO-EX (n=14) and WT-EX (n=15). Separate groups of mice remained sedentary without access to running wheels and were designated FGF21KO-SED (n=9) and WT-SED (n=10). All mice were individually housed in temperature controlled animal quarters (21°C) with a 06:00–18:00 light and 18:00–06:00 dark cycle. All groups were provided ad libitum standard rodent chow (FormulaLab 5008; Purina Mills, Brentwood, MO). Running wheel revolutions were monitored and counted continuously using a Sigma BC 509 bike computer (St. Charles, IL). Running distance was obtained daily between 08:00 and 10:00. Body mass and food consumption were measured on the same day each week throughout the study.

After the 8-week intervention, running wheels were locked for 24 h and mice were fasted for 5 h, anesthetized with pentobarbital sodium (100 mg/kg) and then exsanguinated by removal of the heart. Blood was

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collected via cardiac puncture within 10min of mice being anesthetized (pentobarbital).

Body composition

Fat and lean mass were measured using an EchoMRI 4in1-1100 analyzer (EchoMRI; Houston, TX). Visceral WAT (epididymal), subcutaneous WAT (inguinal region) and BAT (interscapular region) fat pads were removed, weighed and fixed in formalin or flash-frozen in liquid nitrogen until further analysis.

RNA isolation, q-PCR

BAT and WAT (from visceral and subcutaneous regions) samples were homogenized in TRIzol solution using a tissue homogenizer (TissueLyser LT, Qiagen, Valencia, CA). Total RNA was isolated according to the Qiagen’s RNaseasy lipid tissue protocol and analyzed using a Nanodrop spectrophotometer (Thermo Scientific) to assess purity and concentration. First-strand cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed as previously described (Padilla et al. 2013, Crissey et al. 2014) using the ABI StepOne Plus sequence detection system (Applied Biosystems). Primer sequences were designed using NCBI Primer Design tool and purchased from IDT (Coralville, IA). Gapdh and 18s were used as housekeeping control genes and mRNA expression values are presented relative to the WT-SED group. Forward and reverse primer sequences are provided in Table 1.

Fasting blood parameters

Circulating fasting plasma levels of glucose, lipids, FGF21 and adiponectin were assessed as previously described and reported (Fletcher et al. 2016); those data are also included in Table 2 because of their relevance to the new AT data reported herein. Homeostatic model assessment of insulin resistance (HOMA-IR) (Matthews et al. 1985) and AT insulin resistance (Adipo-IR), calculated as fasting NEFA×fasting insulin (Lomonaco et al. 2012), were used to assess insulin resistance.

Western blotting

Triton X-100 tissue lysates were used to produce Western blot-ready Laemmli samples. Protein samples (10µg/ lane) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed with primary antibodies: oxidative phosphorylation (OxPhos) complexes I through V of the electron transport chain (ETC) (1:2000, MitoProfile Total OxPhos Rodent WB Antibody Cocktail, MitoSciences; Eugene, OR), 5’AMP-activated protein kinase (AMPK) (1:1000) and phospho-AMPK at Threonine 172 and Serine 485 residues (1:1000, Cell Signaling), protein kinase B (1:500, Cell Signaling), phosphorylation at Serine 473 (1:250, Cell Signaling), GLUT4 (1:1000, Cell Signaling) and

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**Table 1** qPCR primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
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<tr>
<td>18s</td>
<td>TCA AGA ACG AAA GTC GGA GG</td>
<td>GGA CAT CTA AGG GCA TCA C</td>
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<tr>
<td>Gapdh</td>
<td>CCA GCT ACT CGC GGC TTT A</td>
<td>GAG GGC TGC AGT CCG TAT TT</td>
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<tr>
<td>Fgf21</td>
<td>GTA CCTT CTA CAC AGA TGA C</td>
<td>CAT CTA CCA CGT TCC CAT CCT</td>
</tr>
<tr>
<td>Tnga</td>
<td>CTA TGG CTC AGC CTC TTC TC</td>
<td>ACT TGG GGA AAC TCT CAG GC</td>
</tr>
<tr>
<td>Tcca</td>
<td>TTC AGT TGC CTT CTT GGG AC</td>
<td>CAT TTT GGA AAC TTC TCA TCC</td>
</tr>
<tr>
<td>Leptin</td>
<td>CTT ATT GAT GGG TCT GCC CG</td>
<td>AGT CTC CTC TCC GGA CTT GT</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>GCC ATC ATC TTT CAC AGC AGG</td>
<td>TGA GGG CTA CCA TCA GAG AC</td>
</tr>
<tr>
<td>Cd11c</td>
<td>ATG CCA AGT TGC TCT GGC TTC AT</td>
<td>CAT CTT CTT GAC TCT TAC AG</td>
</tr>
<tr>
<td>Cd8</td>
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<td>GAG CCA GTG CAA AGG TGA CA</td>
</tr>
<tr>
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<td>GAG GAC CAT GGG TGA CTT CT</td>
</tr>
<tr>
<td>Ifngamma</td>
<td>AGC AAG GGC AAA AAG GAT GC</td>
<td>CCT TGG TGC ATG AAA CTC CT</td>
</tr>
<tr>
<td>F22phox</td>
<td>ACT CTA TCG CTC AGT GGG T</td>
<td>TCA TTT GAT GCT TGG CTC C</td>
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<td>Ddit3</td>
<td>ATG TGG AAG ATG AGC GGG TG</td>
<td>AAG CTT CAC CAC AGA GGT CAG</td>
</tr>
<tr>
<td>Ucp-1</td>
<td>CAC GGG GAC CAA CAG TGC TT</td>
<td>TGG AAG ACT CTC TCC TCA GGT</td>
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<tr>
<td>Prdm16</td>
<td>CAG CAC GTG GAA GCC ATT C</td>
<td>ACA GTA AAT GGC AGG GGA CG</td>
</tr>
<tr>
<td>Pgc1a</td>
<td>CCC TGC CAT TGT TAA GAC C</td>
<td>GCG TGC ATC CCG TGG TA</td>
</tr>
<tr>
<td>Cidea</td>
<td>TGC TCT TCT GTA TCG CCC AGT</td>
<td>TGC GC TGC TCC TGC TTT C</td>
</tr>
<tr>
<td>Lpl</td>
<td>GAG ACT CAG AAA AAG GTC ATC</td>
<td>GCC GTG TTA AGG AAT CTG CTG</td>
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<td>Irs-1</td>
<td>GAT CGT CAA TAG CGT AAC TG</td>
<td>GTC TTC AAA GAA CCA AGA TGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATC GTA CCA TCT ACT GAA GAG</td>
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beta-actin (1:2000, Cell Signaling). Intensity of individual protein bands was quantified using FluoroChem HD2 (AlphaView, version 3.4.0.0) and were expressed as a ratio to housekeeping band, beta-actin.

**Histology**

Formalin-fixed visceral WAT and BAT samples were processed through paraffin embedment, sectioned at 5 µm and stained with hematoxylin and eosin (i.e., H&E) for morphometric determinations, as previously described (Padilla et al. 2013). Sections were evaluated using an Olympus BX60 photomicroscope (Olympus, Melville, NY) and photographs were taken at 20× magnification via Spot Insight digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Adipocyte size was calculated based on 100 adipocytes/animal obtained from three 20× fields per animal. Briefly, cross-sectional areas of the adipocytes were obtained from perimeter tracings using ImageJ software as performed previously (Wainright et al. 2015). All procedures were performed by an investigator who was blinded to the experimental conditions.

**Statistics**

Two-way analysis of variance was performed using SPSS, v21 to assess differences among groups for genotype and exercise main effects, as well as genotype × exercise interactions. Post hoc Tukey comparison was utilized to assess between-group differences if genotype by exercise interactions showed statistical significance. For such post hoc analyses, between-group differences are indicated by ‘a’ when different from all other groups, ‘b’ when different compared to WT-SED and ‘c’ when different compared to FGF21KO-EX. All data are presented as means ± standard error of the mean (s.e.); P ≤ 0.05 was considered statistically significant.

**Results**

### Genotype and exercise impact plasma metabolite profile

As previously reported (Fletcher et al. 2016), no differences existed in running distance between FGF21KO-EX and WT-EX groups, and both groups experienced an increase in energy intake and relative cardiac tissue mass, suggestive of similar training adaptations. However, compared to WT-EX, FGF21KO-EX animals weighed more, had a higher total body fat percentage and consumed more total energy (Table 3). As shown in Table 2, FGF21KO animals also had greater fasting serum levels of total cholesterol, glucose and insulin; whereas no genotype differences were observed for triglycerides (TG), NEFAs or the insulin-sensitizing adipokine, adiponectin. In both genotypes,

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Serum values.</th>
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<tr>
<td><strong>Variable</strong></td>
<td>WT-SED</td>
</tr>
<tr>
<td>FGF21, pg/mL</td>
<td>892.5 ± 199.63</td>
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<tr>
<td>Insulin, ng/mL</td>
<td>1.23 ± 0.10</td>
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<tr>
<td>Glucose, mg/dL</td>
<td>280.2 ± 10.75</td>
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<tr>
<td>Adiponectin, µg/mL</td>
<td>21.38 ± 1.05</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>61.06 ± 4.69</td>
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<tr>
<td>Cholesterol, mg/dL</td>
<td>95.37 ± 4.96</td>
</tr>
<tr>
<td>FFA, µM</td>
<td>769.44 ± 53.55</td>
</tr>
</tbody>
</table>

*Significant genotype main effect (P ≤ 0.05); †Significant exercise main effect (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Animal characteristics.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
<td>WT-SED</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.3 ± 1.2</td>
</tr>
<tr>
<td>Running distance, km/day</td>
<td>NA</td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Lean mass, g</td>
<td>22.9 ± 0.6</td>
</tr>
<tr>
<td>Percent body fat, %</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Food consumption, g/week</td>
<td>25.8 ± 0.2</td>
</tr>
<tr>
<td>Feeding efficiency</td>
<td>0.024 ± 0.0004</td>
</tr>
</tbody>
</table>

*Significant genotype main effect (P ≤ 0.05); †Significant exercise main effect (P ≤ 0.05).
EX reduced TG, NEFA and adiponectin levels. No FGF21 protein was detected in serum of FGF21KO mice and EX did not affect circulating levels of FGF21 in WT mice.

Exercise improves adipose tissue insulin sensitivity in FGF21KO mice

Based on HOMA-IR values, FGF21KO mice were significantly more insulin-resistant than WT controls (Fig. 1A) and EX had a tendency \((P=0.112)\) to improve HOMA-IR in both genotypes. Similarly, FGF21KO mice had significantly greater Adipo-IR (a surrogate index of AT insulin resistance \((\text{Lomonaco et al. 2012})\), and EX significantly reduced Adipo-IR for both genotypes (Fig. 1B). Additionally, EX increased epididymal WAT insulin receptor substrate 1 \((\text{Irs1})\) expression (Fig. 1C). However, gene expression of the enzyme necessary for insulin-mediated lipid uptake into AT, lipoprotein lipase \((\text{Lpl})\), was not significantly altered (Fig. 1C).

Phosphorylated (i.e., activated form of) AKT content was significantly upregulated in visceral WAT in the FGF21KO mice (Fig. 1D); whereas GLUT4 protein content was significantly elevated in all three FGF21KO mice groups compared to WT (Fig. 1E). Neither of those proteins were significantly affected by EX in any of the three depots.

In concordance with greater total adiposity, FGF21KO mice had significantly greater visceral and subcutaneous WAT depot weights compared to WT mice, while exercise decreased weights to similar values as WT-SED, but not to the extent of WT-EX (Fig. 2A and B). The significant genotype \(\times\) EX interaction for both subcutaneous and visceral WAT depots is indicative of a more robust adiposity-reducing effect of EX in the FGF21KO animals. Morphometric analysis of histologic sections from the visceral WAT depot revealed mean adipocyte size was greater for the FGF21KO compared to WT mice and was reduced with EX in both genotypes (Fig. 2C and D).

Figure 1
Influence of FGF21 ablation and/or exercise on insulin resistance and WAT. (A) Homeostasis model assessment of insulin resistance (HOMA-IR), (B) adipose tissue insulin resistance (Adipo-IR), (C) EPI Irs1 and EPI Lpl gene expression, (D) phospho-AKT:AKT protein content and representative Western blots below, and (E) GLUT4 protein content with representative Western blots below. EPI, epididymal (visceral); EX, exercise; FGF21KO, FGF21 knockout; SED, sedentary; SQ, subcutaneous (inguinal); WT, wild-type. Values are means ± standard error (s.e.) \((n=9–15)\). *Significant genotype main effect \((P\leq 0.05)\); †Significant exercise main effect \((P\leq 0.05)\).
WAT inflammation is increased in FGF21KO mice and exercise rescues this phenotype

As shown in Fig. 3A and B, FGF21 mRNA was not detected in AT of KO animals (or liver; data not shown), validating the model. Corresponding with their greater adiposity, FGF21KO mice had greater leptin expression in both visceral and subcutaneous WAT. The inflammatory markers monocyte chemoattractant protein-1 (Mcp-1), tumor necrosis factor alpha (Tnfa), the marker expressed on inflammatory/M1 macrophages (Cd11c) and the T cell marker Cd8 were all significantly upregulated in visceral WAT of FGF21KO mice relative to WT controls. Consistent with the previously reported anti-inflammatory effects of exercise in WAT, EX reduced the inflammatory profile of both visceral and subcutaneous WAT independent of genotype (Fig. 3).
Effects of FGF21 ablation and exercise on mitochondrial characteristics of WAT

As AT mitochondrial metabolism is potentially affected by exercise, we assessed markers of mitochondrial content and function in subcutaneous and visceral WAT. Uncoupling protein 1 (Ucp1) mRNA was virtually undetectable in subcutaneous and visceral WAT of FGF21 KO mice. For Pgc1alpha, which induces mitochondrial biogenesis (Fletcher et al. 2016), a significant genotype × EX interaction was observed in both WAT depots (Fig. 3). That is, in both visceral and subcutaneous WAT, FGF21KO animals had lower Pgc1alpha expression, suggesting that lack of FGF21 may reduce mitochondrial biogenesis in WAT; this effect of FGF21 ablation was partially rescued by EX. Interestingly, EX affected WT mice in the opposite way; they experienced (in both WAT depots) an EX-mediated reduction in Pgc1alpha and a similar, yet non-significant, trend with Ucp1. Similarly, Prdm16, the cell fate dictating protein previously shown to induce beige adipogenesis, was reduced with EX in subcutaneous WAT but the effect was not significant in visceral WAT (Fig. 3).

In visceral WAT, EX increased mitochondrial citrate synthase activity, but only in WT mice. In fact, EX actually reduced citrate synthase activity in FGF21KO mice (Fig. 4A). As shown in Fig. 4B, in both genotypes, EX tended to reduce ETC subunit content (COXI, \( P = 0.110 \); COXII, \( P = 0.008 \); COXIII, \( P = 0.041 \); COXIV, \( P = 0.008 \), COXV, \( P = 0.192 \)) in visceral WAT. Similarly, in both genotypes, EX increased AMPK inhibitory activity (Fig. 4C), as indicated by increased serine phosphorylation. In subcutaneous WAT, we noted no differences between groups in citrate synthase activity (Fig. 4D); COXIV content (Fig. 4E), a validated marker of mitochondrial content (Sun et al. 2015) or AMPK content or phosphorylation status (i.e., activity) (Fig. 4F). In subcutaneous WAT, ATP synthase (i.e., COXV) content was increased in FGF21KO mice. EX increased ETC subunit COXI in WT only, and COXIII in both genotypes.

FGF21-null mice display an abnormal BAT phenotype, which is restored with exercise training

Similar to WAT depots, interscapular BAT mass was greater in FGF21KO compared to WT mice and EX reduced total interscapular BAT in both genotypes (Fig. 5A). When expressed relative to total body fat, as measured by EchoMRI, FGF21KO had lower relative BAT, assessed by the equation (BAT mass/(body mass × body fat percentage)) × 100, which was almost fully restored with EX (Fig. 5B). Histological analysis suggested that the increased BAT mass in FGF21KO-EX (and SED) mice was due to increased ‘whitening’, based on a visual increase in lipid droplet size in both FGF21KO-EX and SED mice compared to their respective controls (Fig. 5C). Since recent work revealed an important, lipoprotein lipase (LPL)-dependent role of FGF21 in accelerating lipoprotein catabolism in BAT (Schlein et al. 2016), the BAT-whitening phenotype observed in the FGF21KO caused us to question whether impaired Lpl-mediated lipoprotein catabolism may have played a role. Indeed, we found a suppression in Lpl gene expression in the FGF21KO compared to WT mice (Fig. 5D). EX did not significantly affect BAT Lpl gene expression, with a tendency to reduce it in the FGF21KO. Consistent with the observation of increased BAT whitening, leptin, the classic adipokine that increases in WAT with adipocyte expansion, was significantly upregulated in KO animals, as were several inflammatory and pro-oxidant genes that are also known to increase in WAT with obesity (i.e., Mcp-1; Tnfa; Cd11c; P22phox, Fig. 5E). Remarkably, all those changes observed in FGF21KO-SED mice were completely normalized by EX, which had a similar protective effect in the WT animals. EX also reduced the classic macrophage marker, Erm-1 and the cytokine Il-6, which were not affected by FGF21 ablation. Since Tnfa, a major inflammatory cytokine, is known to cause adipocyte insulin resistance in WAT via impaired Irs1 activity (Hotamisligil et al. 1996), we measured Irs1 gene expression in BAT to determine if there was evidence of inflammation-induced insulin resistance or protective effects of EX in this regard. An elevation in Irs1 may be indicative of adipocyte compensation for insulin resistance; thus, reduced levels may indicate improvements in insulin signaling. Consistent with this hypothesis, EX reduced both BAT Tnfa and Irs1 expression; this EX effect was observed in both WT and KO groups (Fig. 5D). These findings validate previous work showing EX training reduces inflammation in WAT (Vieira et al. 2009a, Linden et al. 2014) and BAT (Xu et al. 2011, Welly et al. 2016) and demonstrate that while inflammation is increased in the AT of FGF21KO mice, the anti-inflammatory effects of EX do not require FGF21.

BAT mitochondrial function was assessed by measuring mitochondrial enzyme (i.e., citrate synthase) activity (Fig. 6A), ETC subunit protein content (Fig. 6B), AMPK content and phosphorylation state (Fig. 6D). Here, we found that lack of FGF21 significantly reduced the amount of two important ETC subunits, COXIII and COXIV. Similar to subcutaneous WAT, gene expression of Prdm16 was increased in BAT of FGF21-KO mice, yet
Figure 4
Influence of FGF21 ablation and/or exercise on WAT mitochondrial content and activity. (A) EPI citrate synthase activity, (B) EPI Ox Phos content with representative Western blot images, (C) EPI AMPK and phosphorylated AMPK at Serine 485 and Threonine 172 residues with representative Western blot images, (D) SQ citrate synthase activity, (E) SQ Ox Phos content with representative Western blot images and (F) SQ AMPK and phosphorylated AMPK at Serine 485 and Threonine residues with representative Western blot images. AMPK, 5′AMP-activated protein kinase; EPI, epididymal (visceral); EX, exercise; FGF21KO, FGF21 knockout; Ox Phos, mitochondrial oxidative phosphorylation subunit complexes; SED, sedentary; SQ, subcutaneous (inguinal); WT, wild-type. *Significant genotype main effect ($P \leq 0.05$); †Significant exercise main effect ($P \leq 0.05$); a Significantly different compared to all other groups based on significant genotype × exercise effect followed by post hoc Tukey’s tests ($P \leq 0.05$); b Significantly different than WT-SED based on significant genotype × exercise effect followed by post hoc Tukey’s tests ($P \leq 0.05$); c Significantly different than FGF21KO-EX based on significant genotype × exercise effect followed by post hoc Tukey’s tests ($P \leq 0.05$).
normalized with EX (Fig. 5E). It should be noted that BAT adaptive thermogenic activity was not directly assessed. Taken together, these results indicate that loss of FGF21 increases BAT and WAT inflammation, and may adversely affect normal mitochondrial adaptations to exercise. Additionally, the present data do not support exercise-mediated browning of WAT in mice following 8 weeks of voluntary wheel running.

Discussion

FGF21 is an endocrine hormone that is mainly produced in the liver, but is expressed in several tissues including skeletal muscle, pancreas and AT (Perez-Marti et al. 2016). While the mechanism(s) behind FGF21’s potent insulin-sensitizing effects are not completely understood, its specific actions are thought to be key in predicting its systemic benefits (Adams et al. 2012, Gomez-Hernandez et al. 2016, Samms et al. 2016). Exercise training has strikingly similar actions, profoundly affecting AT by reducing inflammation and improving mitochondrial metabolism (i.e., improving ‘immunometabolism’) (Thompson et al. 2012, Vieira-Potter et al. 2015), yet the mechanisms are not well understood. Intriguingly, a vast majority of studies demonstrate changes in circulating FGF21 levels with exercise. However, the inconsistencies in the magnitude and direction of change make it difficult to define the extent to which FGF21 may contribute to the metabolic adaptations associated with exercise. Although acute exercise increased circulating and skeletal muscle FGF21 levels in some studies (Kim et al. 2013, Slusher et al. 2015, Hansen et al. 2016, Tanimura et al. 2016), it induced no change or a reduction in circulating FGF21 in others (Taniguchi et al. 2016).

Thus, its role in exercise-mediated browning of WAT (Stanford et al. 2015), a process associated with both reduced inflammation (Liu et al. 2015) and increased mitochondrial function (Bae et al. 2014), requires further investigation. Here, we tested whether FGF21 is required
FGF21 and exercise on AT immunometabolism

for exercise-mediated immunometabolic adaptations in AT. To this end, we used FGF21KO mice to determine if FGF21 is necessary for the immunometabolic adaptations due to exercise training in WAT and/or BAT, demonstrating for the first time that absence of FGF21 under normal dietary conditions leads to a dysfunctional AT phenotype characterized by inflammation and impaired mitochondrial oxidative metabolism. Importantly, this unhealthy phenotype was largely rescued with exercise training, indicating that FGF21 is dispensable for exercise-induced anti-inflammatory effects in AT.

In agreement with previous studies (Badman et al. 2009, Laeger et al. 2014), we found that FGF21KO mice have increased adiposity, which is accompanied by an overall impaired metabolic profile. Although energy expenditure was not directly assessed, FGF21KO animals consumed more energy, suggesting that their greater adiposity was at least partially due to hyperphagia. Voluntary wheel running reduced all measures of adiposity in both WT and FGF21KO animals, and FGF21KO were no less responsive to those adiposity-reducing effects. Recently, Loyd and coworkers exposed FGF21KO mice to chronic high-fat feeding and voluntary wheel running, and also demonstrated that the effects of exercise on body weight and adiposity occur independent of FGF21 (Loyd et al. 2016). Increased adiposity and adipocyte size in the FGF21KO-SED group were associated with a higher Adipo-IR value, a surrogate index of AT insulin resistance (Lomonaco et al. 2012). Consistently, expression of pro-inflammatory and endoplasmic reticulum (ER) stress markers was increased in visceral WAT from FGF21KO-SED mice. Reduced Adipo-IR, which associated with increased Irs1 expression and decreased inflammatory/ER stress gene expression in WAT, regardless of genotype, further demonstrating that exercise’s ability to improve WAT phenotype does not require FGF21.

Exercise-associated reductions in WAT inflammation have been previously reported; however, the majority of these studies were performed in obese animals (Bradley et al. 2008, Vieira et al. 2009a, Welly et al. 2016). Here, we demonstrate that the anti-inflammatory effects of exercise also occur in WAT and BAT of otherwise healthy, but previously sedentary, chow-fed mice. While FGF21KO-SED WAT and BAT had a more pro-inflammatory phenotype compared to WT-SED mice, it is unclear if that was a direct effect of FGF21 ablation or secondary to the increase in adiposity observed in the KO mice. However, emerging data suggest a direct role for FGF21 in alleviating ER stress and inflammation (Wang et al. 2014, Guo et al. 2016, Yu et al. 2016). In order to interrogate further whether the
inflammatory changes observed in the KO mice were dependent or independent of the increase in adiposity, we statistically adjusted for the effect of body weight on all inflammatory outcomes (Supplementary Table 1, see section on supplementary data given at the end of this article). We found that many, yet not all, of the differences in inflammatory genes were no longer significantly elevated in the KO compared to WT mice, suggesting that the elevated adiposity in the KO contributed significantly to their greater WAT and BAT inflammation. However, even after this statistical adjustment, KO maintained greater levels of visceral WAT Cdd8 (an inflammatory T cell marker associated with insulin resistance (Nishimura et al. 2009) gene expression and tended to (P=0.053) maintain greater Tnfa (an inflammatory cytokine known to secreted by inflammatory macrophages and impair adipocyte insulin signaling (Hotamisligil et al. 1996)) levels; these findings support that the absence of FGF21 does have adverse inflammatory effects on AT independent of body weight. While it is likely that the effect of exercise to reduce AT inflammation was at least somewhat driven by its ability to reduce adiposity, even after the body weight adjustment, exercise-mediated reductions in WAT and BAT Leptin gene expression remained statistically significant, as did the exercise-mediated reduction in WAT macrophage gene expression (i.e., Erm-1), confirming previous reports of exercise having direct anti-inflammatory effects in AT (Castellani et al. 2014, Peppler et al. 2017).

Accumulating evidence supports important relationships between mitochondrial and immune function suggesting that impaired mitochondrial health associates with greater inflammation (Vamecq et al. 2012, Qatanani et al. 2013, Hahn et al. 2014). Although lean AT is characterized by the absence of inflammation and increased mitochondrial function (Flachs et al. 2013), obese/sedentary AT is typified by a pro-inflammatory phenotype and impaired mitochondrial activity (Okamoto et al. 2007). Meanwhile, exercise improves WAT mitochondrial content and function (Wang et al. 2014, Stanford et al. 2015) and reports have implicated FGF21 in mediating mitochondrial biogenesis via regulation of Pgc1alpha (Okamoto et al. 2007, Stanford et al. 2015). Thus, mitochondria-related markers were assessed in WAT and BAT to determine the potential impact of loss of FGF21 on the ability of exercise to affect these parameters. Consistent with our previous observation that hepatic mitochondrial function is impaired in FGF21KO mice and almost completely restored by exercise training (Fletcher et al. 2016), our current findings revealed important roles of both FGF21 and exercise in mediating AT mitochondrial adaptations.

In the absence of FGF21, BAT took on a phenotype more similar to WAT, including increased inflammation (e.g., increased Tnfa, Mcp-1, P22phox and Cdl1c gene expression) and greater lipid deposition. Exercise decreased that inflammatory profile in both WT and KO mice. However, adjusting for total body weight caused most of those inflammatory improvements to lose statistical significance (Supplementary Table 1). Importantly, AT inflammation is known to adversely affect adipocyte insulin signaling, which contributes to systemic insulin resistance. While most work on inflammation-induced insulin resistance has been done on WAT, BAT is also insulin sensitive and its presence contributes to systemic protection against insulin resistance (Townsend & Tseng 2012). Thus, BAT inflammation may adversely affect brown adipocyte insulin sensitivity and contribute to systemic insulin resistance, whereas FGF21 and exercise may both improve systemic insulin sensitivity by mitigating inflammation in WAT and BAT. Indeed, we found that exercise reduced BAT Tnfa and Irs1 gene expression, whereas Tnfa is known to cause adipocyte insulin resistance via its inhibitory actions on Irs1 (Hotamisligil et al. 1996). In terms of BAT mitochondrial assessments, KO had significantly lower mitochondrial oxidative phosphorylation complexes III and IV (i.e., COXIII, IV protein content). Although the main effect of genotype on COXIII was not statistically significant following body weight adjustment, the suppressed BAT COXIV in KO remained even after this adjustment, suggesting a direct protective role of FGF21.

In conclusion, absence of FGF21 results in increased adiposity, AT insulin resistance and inflammation in both WAT and BAT. Importantly, exercise largely normalized the dysfunctional AT phenotype in FGF21KO animals. These new findings reveal the importance of FGF21 in maintaining a healthy AT phenotype under sedentary conditions, while revoking the hypothesis that the anti-inflammatory effects of exercise in AT require FGF21.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-17-0190.

**Declaration of interest**
J W Perfield II is a paid employee of Eli Lilly and Company and may own company stock or possess stock options. The remaining authors have no conflicts of interest to disclose.
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