Mitochondrial myopathy induces a starvation-like response

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Mitochondrial respiratory chain (RC) deficiency is among the most common causes of inherited metabolic disease, but its physiological consequences are poorly characterized. We studied the skeletal muscle gene expression profiles of mice with late-onset mitochondrial myopathy. These animals express a dominant patient mutation in the mitochondrial replicative helicase Twinkle, leading to accumulation of multiple mtDNA deletions and progressive subtle RC deficiency in the skeletal muscle. The global gene expression pattern of the mouse skeletal muscle showed induction of pathways involved in amino acid starvation response and activation of Akt signaling. Furthermore, the muscle showed induction of a fasting-related hormone, fibroblast growth factor 21 (Fgf21). This secreted regulator of lipid metabolism was also elevated in the mouse serum, and the animals showed widespread changes in their lipid metabolism: small adipocyte size, low fat content in the liver and resistance to high-fat diet. We propose that RC deficiency induces a mitochondrial stress response, with local and global changes mimicking starvation, in a normal nutritional state. These results may have important implications for understanding the metabolic consequences of mitochondrial myopathies.

INTRODUCTION

Skeletal muscle is the organ with the highest mass in the body, a major user of oxidative ATP and highly dependent on mitochondrial respiratory chain (RC) function, as indicated by a wide spectrum of mitochondrial disorders involving the muscle. Progressive dysfunction of the RC is a common cause of adult-onset myopathies and a key diagnostic finding in RC deficiency is the presence of cytochrome c oxidase (COX)-deficient fibers in a skeletal muscle biopsy sample. The physiological consequences of progressive RC dysfunction are not well known.

Familial progressive external ophthalmoplegia (PEO) with multiple mtDNA deletions is a typical example of a progressive adult-onset RC deficiency (1,2). We and others have reported underlying defects in a number of proteins: adenine-nucleotide translocator 1, mtDNA polymerase gamma, mtDNA helicase Twinkle and ribonucleotide reductase.

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Gene Accession Description Assigned function or pathway Fold change P-value

**Upregulated genes**

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**Table 1.** Microarray data (P ≤ 0.05) of up- (>2-fold) and downregulated genes (>1.5-fold) in Deletor muscle

Genes containing the AARE/CEBP module are given in bold.

subunit p53R2 (3–7). In familial PEO disease, the primary nuclear gene mutation induces secondary deletion formation in mtDNA (1,2). The deletions accumulate with age and manifest as disease symptoms when the mutant mtDNA exceeds a threshold that causes RC deficiency. PEO patients typically have 5–20% COX-negative fibers in their muscle (8).

We have previously described a mouse model for PEO, the Deltor mouse (9). These transgenic mice express a dominant PEO mutation, a 13 amino acid duplication in the mitochondrial helicase Twinkle, under the ubiquitous β-actin promoter. At 1 year of age, the Deletors present with COX-deficient muscle fibers as well as with progressive subtle accumulation of multiple mtDNA deletions in the muscle and brain, but have a normal lifespan. The Deltor mouse therefore replicates the key features of late-onset mitochondrial myopathy, and is a unique model to study pathogenesis of subtle progressive RC dysfunction, as no other patient mutation expressing models for late-onset mitochondrial myopathy have been reported. We studied here the consequences of subtle progressive RC deficiency for skeletal muscle, and identified both local and global responses, closely linked to starvation response.

**RESULTS**

Transcriptional amino acid and lipid starvation response in Deletor muscle

We performed gene expression profiling of *quadriceps femoris* muscle of 20- to 24-month-old Deltor mice, with late-onset progressive mitochondrial myopathy. Table 1 lists the most up- and downregulated transcripts. The array data were replicated by quantitative real-time polymerase chain reaction (qRT-PCR); (Supplementary Material, Fig. S1). The upregulated transcripts included genes previously linked to amino acid starvation response (10): methyltetrahydrofolate dehydrogenase 2 (Mthfd2; +3.8-fold), splicing factor proline/glutamine rich (Spfq; +3.1-fold), asparagine synthetase (Asns; +1.7-fold) and phosphoserine aminotransferase (Psat1; +2.2-fold) (Supplementary Material, Fig. S1). Furthermore, expression levels of two transcripts involved in the regulation of lipid metabolism were significantly altered. Fibroblast growth factor 21 (Fgf21; +3.0-fold) was recently described as a major regulator of starvation response, especially involved in lipid mobilization (11,12). Periodic-2 was significantly downregulated (−1.6-fold), and is known to be involved in regulation of the circadian rhythm of muscle lipid metabolism.

To find putative common regulatory elements for the upregulated genes, we studied their promoter regions by bioinformatic tools. We found typical amino acid response elements (AAREs), conserved across species, in the proximal regulatory regions of 6 out of 15 of the >2-fold induced genes (Mthfd2, Fgf21, Map4, Cul5, Rc3h2 and Luc7l2) and in classical amino acid response genes Asns and Trib3 (Fig. 1). The AAREs in these regions are located in a composite site that also binds CCAAT/enhancer binding protein (CEBP), forming a functional AARE/CEBP module. The module is found in 1.98% of vertebrate promoter sequences, being 40 times enriched in the upregulated genes in the Deltor muscle. The AARE is known to bind ATF factor family members, of which Atf5...
was mildly increased (±1.5-fold) in the Deletor muscle, and to stimulate the expression of the target genes upon limited nutrition (13). These data suggested that mitochondrial myopathy induces a starvation-like response, involving both lipid and amino acid metabolism.

Amino acid metabolism in Deletors

To study whether abnormal amino acid levels underlie the induction of the starvation response, we measured the free amino acid levels by metabolomics tools. We have recently shown that Deletor mouse serum has increased levels of most amino acids, probably as a sign of muscle protein breakdown, and that these aberrant levels are normalized by a ketogenic, high-fat (HF) diet (14). We now studied the skeletal muscle amino acid levels and found that the Deletor and control muscle had similar levels of amino acids, except for a 1.7-fold increased amount of serine ($P = 3.1 \times 10^{-2}$) in males and a 1.5-fold increased amount of alanine ($P = 0.022$) in females (Supplementary Material, Fig. S2A and B). The increased amino acid levels in the serum and normal levels in the skeletal muscle suggest that the lack of specific amino acids did not cause the transcriptional response in the muscle.

**AKT1 activation, but not PGC1α, is involved in the mitochondrial stress response**

Pathway analysis of the expression data (Supplementary Material, Tables S1 and S2), utilizing Ingenuity Pathway Analysis, indicated that among the most significantly altered pathways were several pathways involving phosphatidylinositol 3-kinase (PI3K) signaling, including Akt/PI3K ($P = 3.2 \times 10^{-2}$). PI3K/Akt1 activation is linked to a plethora of metabolic and survival pathways, including nutrient sensing, in different cell types (15). Muscle-specific Akt1 overexpression in mice was previously shown to induce Fgf21 expression, whereas the PI3K inhibitor LY294002 reduced its expression in cultured myocytes (16). In a western blot analysis, total AKT levels did not differ significantly between wild-type and Deletor muscle, but the phosphorylated forms of amino acids 308 and 473 of AKT1, necessary for AKT activation, were significantly increased in the Deletor (Fig. 2A and B).

PGC1α has been shown to negatively regulate Fgf21 expression in liver (17). We found that Pgc1α, Pgc1β, (Fig. 2C) Ppara or Ppary mRNA or their target genes (microarray) were not changed in the Deletor. Furthermore, in mice that overexpress Pgc1α, with massive proliferation of muscle mitochondria (18), Fgf21 and Mthfd2 expression levels were similar to the wild-type (Fig. 3I and L), indicating that Fgf21 and Mthfd2 are not induced because of mere Pgc1α overexpression or mitochondrial proliferation. These data support the role of Akt1 signaling in the stress response induced by mitochondrial dysfunction in skeletal muscle.

**Mthfd2 and Fgf21 induction follows the progression of RC deficiency**

To follow up the expressional response upon disease progression, we studied the expression of Mthfd2 and Fgf21 in Deletors of different ages. By qRT-PCR, we found that Fgf21 expression followed closely the number of COX-negative muscle fibers and disease progression (Fig. 3A). The expression levels of Fgf21 in Deletor liver, adipose tissue or brain were not increased (Fig. 3A), suggesting a
3-fold higher Fgf21 levels in Deletor plasma compared with body production in the liver (11,12,21,22). We detected fasting, and to mobilize stored lipids and induce ketone secreted from the liver or adipose tissue upon prolonged described metabolic hormone that has been found to bevation, in a normal nutritional state. Fgf21 is a recently interpreted the mitochondrial RC defect as a state of star-

According to the expression data, the skeletal muscle was
mitochondrial myopathy

Fgf21 and Mthfd2 are induced in Cox10-knock-out mice

To investigate whether Fgf21 or Mthfd2 induction was a common response to muscular RC deficiency, we obtained samples of other mouse models with mitochondrial dysfunction. The muscle-specific inactivation of COX assembly factor Cox10 (Cox10-KO) leads to severe COX-deficiency and progressive mitochondrial myopathy (23). Simultaneous muscle-specific overexpression of the transcriptional co-activator Pgc1α, which induces mitochondrial biogenesis, considerably delays the development of the COX negative fibers (18). We found that Fgf21 mRNA expression was 2.2-fold and Mthfd2 protein 8.5-fold increased in the muscle of Cox10-KO mice, but was similar to the wild-type in the [Cox10 x Pgc1α] and Pgc1α overexpressor muscles—supporting the finding that their induction was linked to RC deficiency (Fig. 3I and L). These data support the conclusion that manifesting RC deficiency in the skeletal muscle induces Fgf21 and Mthfd2 expression.

Metabolic consequences of mitochondrial myopathy in mice

Systemic availability of Fgf21 could play a role in the metabolic alterations that often are associated with mitochondrial diseases, i.e. cachexia or thinness. The effects of hepatic overexpression of Fgf21 have been studied in mice, in which elevated plasma Fgf21 concentrations led at 9 months of age to low weight, low hepatic fat content and small adipocytes (22). Similar consequences followed intravenous Fgf21 administration (24) and elevated Fgf21 serum levels in mice with muscle-specific Akt1 overexpression (16,25). We found similar signs in the Deletors: their muscular Fgf21 levels started to increase at 12 months along with the progression of mitochondrial myopathy, and they weighed significantly less than the wild-type littermates from 14 months of age (wild-type male 35.7 ± 4.4 g, Deletor male 31.1 ± 2.0 g, P = 0.007, n = 8 for both groups). The Deletors had a smaller adipocyte size compared with age-matched controls (50% reduction in D line; 36% reduction in C line) (Fig. 4A, B and E), which was not due to deletions or depletion of mtDNA in white adipose tissue. Deleter livers showed reduced amounts of lipids (Fig. 4C and D). Cholesterol and triglyceride levels were previously found to be unaltered in Deletor plasma (14). We have also previously shown that the Deletors were resistant to HF diet-induced weight gain (14), similar to mice with overexpressed Fgf21 in liver (22), or Akt1 in muscle, both having elevated Fgf21 serum levels (25). This suggested that the resistance to weight gain in Deletors could be muscle induced and linked to Fgf21 expression. Furthermore, we showed previously that a HF diet reduced the number of COX-negative fibers (14). Consistent with those findings, here we show that the Deletors on a HF diet have lower muscle Fgf21 expression than those on a normal diet

Fgf21 hormone is systemically available upon mitochondrial myopathy

According to the expression data, the skeletal muscle was interpreting the mitochondrial RC defect as a state of starvation, in a normal nutritional state. Fgf21 is a recently described metabolic hormone that has been found to be secreted from the liver or adipose tissue upon prolonged fasting, and to mobilize stored lipids and induce ketone body production in the liver (11,12,21,22). We detected 3-fold higher Fgf21 levels in Deletor plasma compared with controls (Fig. 3G). These results, in addition to local immunopositivity colocalizing with RC-deficient fibers and the lack of increased expression in liver and adipose tissue, raise an interesting possibility that Fgf21 could be secreted from the diseased muscle fibers upon RC deficiency.
Mthfd2 transcript levels were also lower after a HF diet (Fig. 4G). The metabolic effects observed in Deletors were not associated with changes in circulating thyroid or growth hormone levels (Supplementary Material, Fig. S2C–E), or altered insulin or glucose tolerance (Fig. 4H–J). We conclude that the mitochondrial myopathy in mice is associated with lipid metabolic alterations, which resemble both consequences of fasting, and findings in other models with increased Fgf21 levels in the serum.

Figure 3. Fgf21 (A–I) and Mthfd2 (J–L) induction follow the progression of RC deficiency in skeletal muscle of mice. (A) Fgf21 mRNA expression levels in Deletor (Del) muscle, the brain, adipose tissue, liver and in the muscle of transgenic mice overexpressing wild-type (WT) Twinkle. COX−, COX-negative fibers; % indicates the percentage of COX-deficient fibers at each time point (m = months). *P < 0.05, **P < 0.005. (B–F) Immunohistochemistry (IHC) with Fgf21 (B) and succinate dehydrogenase (C) antibodies, serial sections (B), (F) and (E). Arrows indicate the muscle fibers which have increased numbers of mitochondria (arrows). (D) Fgf21 immunoreactivity in punctuate vesicles around the nuclei and in the cytoplasm. Inset: magnification of the fiber indicated by the arrow. (E) Deletor muscle IHC without primary antibody. (F) Fgf21 IHC on WT mouse muscle. Scale bars 20 μm (B, C, E and F), 40 μm (D). (G) Plasma Fgf21 concentrations in Deletor mice compared with age-matched WT mice. *P < 0.02 compared with control littermates (WT) (Student’s t-test). (H) Left: western blot of FGF21 in Deletor muscle. Right: quantification of the western signals. The 30 kDa isoform [starvation induced (21)] and 23 kDa (native form) are shown. *P < 0.005 compared with control littermates (Student’s t-test). (I) Fgf21 expression in 3-month-old Cox10-KO skeletal muscle, in [Pgc1α × Cox10-KO] mice and in Pgc1α-overexpressing mice. (J) Mthfd2 mRNA expression levels in Deletor (Del) muscle, the brain, adipose tissue, liver and in the muscle of transgenic mice overexpressing WT Twinkle. COX−, COX-negative fibers; % indicates the percentage of COX-deficient fibers at each time point (m = months). *P < 0.05, **P < 0.005. (K) Mthfd2 western analysis of Deletor muscle, quantified below. (L) Mthfd2 western analysis in 3-month-old Cox10-KO muscle, in [Pgc1α × Cox10-KO] mice and Pgc1α-overexpressing mice.

(Fig. 4F). Mthfd2 transcript levels were also lower after a HF diet (Fig. 4G). The metabolic effects observed in Deletors were not associated with changes in circulating thyroid or growth hormone levels (Supplementary Material, Fig. S2C–E), or altered insulin or glucose tolerance (Fig. 4H–J). We conclude that the mitochondrial myopathy in mice is associated with lipid metabolic alterations, which resemble both consequences of fasting, and findings in other models with increased Fgf21 levels in the serum.

Fgf21 or Mthfd2 expression in normal skeletal muscle is not considerably affected by fasting or a high-fat diet

The functions of Fgf21 or Mthfd2 are unclear in the skeletal muscle, and thus we tested whether they are induced as a general physiological response of healthy skeletal muscle to stress. Fgf21 induction has previously been reported in the livers of mice that are fasted (12) or fed with a ketogenic diet (11). We could replicate the starvation-related induction in the liver after a 24 h fasting period. However, under the same
fasting conditions that induced 200-fold Fgf21 expression in the liver, Fgf21 or Mthfd2 expression in wild-type skeletal muscle was not significantly altered (Fig. 5). A HF diet for 4 days led to 2.5-fold Fgf21 induction in the wild-type skeletal muscle, but 50-fold in the liver (Fig. 5). Twelve months of a HF diet, or strenuous treadmill training for a 6-week period did not induce Fgf21 (Figs. 4F and 5) or Mthfd2 (Figs. 4G and 5) in the muscle of normal mice. These studies indicate that transient depletion of fuel stores in healthy muscle does not induce Fgf21 or Mthfd2 expression, but their activation is linked to mitochondrial RC dysfunction.

**DISCUSSION**

The physiological consequences of mitochondrial RC disorders are not well known. The studies have been hampered by the lack of relevant disease models and because of heterogeneity of mitochondrial patient materials. We studied the skeletal muscle gene expression patterns of the Deletor mouse, manifesting a dominant-progressive late-onset mitochondrial myopathy, with multiple mtDNA deletions and progressive RC deficiency (9). The major new findings of the study were: (i) subtle progressive RC defect in muscle elicits a transcriptional response mimicking starvation, including induction of several transcripts involved in amino acid and lipid starvation response; (ii) AKT1 phosphorylation and transcriptional response is induced, linking the AKT pathway activation to mitochondrial RC dysfunction in muscle; (iii) single RC-deficient muscle fibers induce the expression of the metabolic regulator Fgf21, increased levels of which are also seen in the mouse plasma; (iv) Deletor adipose tissue and liver show changes previously associated with Fgf21 overexpression (11, 12, 22). The starvation response can be compensated with a HF diet, also found previously to reduce the number of morphological changes in mitochondria (14).

We found signs of both local and global metabolic changes as a response to RC deficiency in the skeletal muscle. A specific local signature in the muscle included activation of known amino acid starvation response genes, and new genes sharing AAREs in their regulatory regions. MTHFD2, ASNS, TRIB3 and ATF5 were previously listed to be induced in a data set with transcripts from skeletal muscle of patients with PEO (26). Also, our mouse data showed clear upregulation of Mthfd2 and Asns. MTHFD2, ASNS, TRIB3, CEBP homologous protein (CHOP) and PSAT1 were induced in cybrid cells harboring a MELAS mutation (27), and we also saw upregulation of Mthfd2, Asns and Psat1 in Deletor muscle. Amino acid response and activation of the AARE-regulated transcription factor CHOP has been linked to a specific mitochondrial stress response pathway, known as the mitochondrial unfolded protein response,
inducing expression of mitochondrial chaperone proteins (28). We found an AARE/CHOP module located near the AARE/CEBP module in the Fgf21 promoter, but did not observe activation of Chop transcript, suggesting that the AARE activating transcription factors may partially differ between cultured cells and muscle, although the overall response is highly similar. Our mouse expression data, from a homogeneous inbred genetic background, links together previous findings from human muscle and cultured cells: induction of the amino acid response is part of mitochondrial disease pathogenesis. Furthermore, we were able to show that free amino acid levels in the Deletor mouse plasma were high (14) and in the normal range in the skeletal muscle, indicating that the response was not due to a limitation in specific amino acids. Our previous studies showed that the most severely affected muscle fibers of Deletor mice had increased recycling of mitochondria through autophagy and degradation of myofibrils (9), the latter being similar to human mitochondrial myopathies. As muscle is a major source of protein utilized for ATP production in fasting, it is intriguing to suggest that myofibrillar proteolysis is a consequence of a local pseudostarvation response, induced by a chronic progressive RC defect.

In addition to a amino acid starvation response, both local and global lipid metabolic derangements were found in the Deletor mice. Fgf21 is a recently described metabolic regulator, which lacks a heparin-binding domain present in most other FGF family members, allowing its secretion and hormone-like function (22,29). Fgf21 attracted high interest, when chronic administration was found to lower plasma insulin and improve glucose tolerance in diabetic rodents and non-human primates (22,30). Furthermore, transgenic overexpression of Fgf21 resulted in reduced adipocyte size and resistance to aHF diet (22). Fgf21 knock-out mice showed deranged hepatic ketone body production upon starvation (31). Fgf21 induction in the liver and adipose tissue was dependent on PPAR pathways, when fatty acid oxidation was required in fasting or in adapting to a ketogenic diet (11,12). Therefore, the hormone has been considered to be essential for the regulation of long-term energy balance and metabolism in mice (11,12), and was recently described as the ‘missing link in the biology of fasting’ (32). We found Fgf21 to be induced in the muscle fibers with RC defect, showing an intracellular vesicle-like localization. Furthermore, in an independent mouse model, the muscle-specific Cox10-KO, we identified increased levels of Fgf21 in the muscle. Induction of amino acid starvation pathways and Fgf21 strongly supports the conclusion that the skeletal muscle interprets RC deficiency as a state of starvation, in a normal nutritional state. Whether the initial mitochondrial stress signal is a low ATP level, cAMP, reactive oxygen species, the NAD + NADH ratio, nitric oxide, a specific metabolite, an amino acid or a peptide messenger remains to be established. However, Fgf21 levels were decreased upon a HF diet in the Deletor, along with improving ultrastructural changes in COX-negative muscle and normalizing serum amino acid levels (14), suggesting that the response can potentially be compensated by a low-glucose, high-lipid diet.

We found it extremely interesting that Fgf21, a secreted hormone, was upregulated upon RC deficiency in the skeletal muscle. Also, Fgf21 serum levels were significantly increased in the Deletors, while no induction of expression was found in other tissues except for muscle. It is therefore possible that the serum Fgf21 originated from the muscle. Muscle-secreted hormones have been speculated to exist, with the potential to act on adipose, hepatic or central nervous system tissues (25). Moreover, skeletal muscle has been previously shown to express Fgf21 (16,33), and its availability in serum was previously associated with small adipocyte size, resistance to a HF diet and reduced hepatic lipids (22,24). The Deletor replicates all these findings; therefore, it is an intriguing possibility that skeletal muscle-derived, systemically available Fgf21 would be a mediator of the global lipid metabolic effects in the mitochondrial myopathy mouse. Mitochondrial disorders are associated with lipid metabolic derangements, such as leanness (34), and the potential relevance of the hormone in human disease requires further attention.

The PI3K/Akt pathway was among the most significantly changed pathways in our analysis of transcriptome data from the Deletor muscle, whereas no indication of activated PPAR pathways was found. However, the 308 and 473 phosphorylated forms of AKT1, required for full activation of the kinase, were significantly increased, indicating that Akt signaling was activated in Deletor muscle. Previously, Akt1 overexpression in the skeletal muscle of obese mice caused muscle hypertrophy, which led to global metabolic improvements and reduced fat mass (25), and later these mice were reported to have induced Fgf21 in muscle and serum (16). These findings strongly support the conclusion that AKT1 activation is an upstream event of Fgf21 activation and secretion in RC-deficient muscle.

Mthfd2 has previously been shown to be induced by starvation in cultured myotubes (10) and upon mitochondrial defects (26,27). Mthfd2 catalyzes the conversion of folic acid intermediates and is thought to be necessary for the synthesis of purines to support growth during embryogenesis (35),

Figure 5. Fgf21 and Mthfd2 are not induced in normal skeletal muscle upon temporary energy deprivation. (A–B) WT mouse muscle: Fgf21 and Mthfd2 mRNA expression in the skeletal muscle and liver of normally fed WT mice and those fasted for 24 h; high-fat diet for 4 days (4 d HF); in strenuous treadmill training (rest, runners).
linking it to nucleotide pool maintenance. We suggest that Fgf21 and Mthfd2 are integral parts of the mitochondrial stress response, and both have the potential to serve as markers for mitochondrial RC deficiency.

Our study is the first global expression analysis performed on a mouse model for a late-onset mitochondrial disease, or on mice expressing a true human mitochondrial disease mutation. The homogeneous genetic background of the mice reduced the biological variance, typical for expression analyses of tissues of mitochondrial disease patients. We identified a clear induction of fasting response in RC-deficient muscle, and strong indications of global lipid metabolic derangements. Our results in vivo establish that mitochondrial stress response is activated upon pathogenesis of mitochondrial disorders, and is important for understanding metabolic features of late-onset mitochondrial myopathies, as well as for developing diagnosis and treatment.

MATERIALS AND METHODS

Mouse tissue collection

All animal procedures were performed according to protocols approved by the ethical boards for animal experimentation of the National Public Health Institute and Helsinki University, as well as State Provincial offices of Finland (agreement number STU575A/2004), and all experiments were done in accordance with good practice of handling laboratory animals and of genetically modified organisms. The generation and the phenotypes of the Deletor and the wild-type Twinkle overexpressor mice have been previously described (9,19). The transgenic mice were backcrossed to C57Bl background for 10 generations, and the congeneity was confirmed with the Mouse Medium Density SNP Panel (Illumina). The disease phenotype was confirmed to be the same in the C57Bl background as in the original FVB/N background. Mice from both backgrounds and from two founder lines (C and D) with different transgene insertion sites were used. The amount of overexpression was modest in both lines, mimicking the 1:1 ratio in the equivalent dominant human disease: the ratio of transgene-derived mutant versus native Twinkle was 0.5 in C-line mice and 1.7 in D mice (9). The higher transgene expression level in D mice caused a slightly faster progression of the disease: the number of COX-deficient muscle fibers at 14 months was on average 6.2% in male D mice and 5.0% in male C mice at 18 months. The mice were housed in a humidity- and temperature-controlled environment (21°C, 60% humidity, 12 h light–dark cycle) with free access to chow (Global 18% Protein Rodent Diet, Harlan Teklad) and water. For the HF feeding, the mice were maintained between the age of 3 and 14 months on a HF D05052004 chow (89.5 kcal % fat, 0.1 carbohydrate, 10.4 proteins; Research Diets, Inc.) or control D05052002 chow (11.5 kcal % fat, 78.1 carbohydrate, 10.4 protein).

For the treadmill exercise, Deletor mice and wild-type littermates were randomized into a treadmill training group (motorized Exer-6M Treadmill, Columbus Instruments) or a sedentary group at the age of 1 year. After initial training of the mice, the mice exercised 5 days a week for 6 weeks. The training speed was calculated from maximal speed and represented an equivalent of 75% of VO2max in 12 month-old male C57BL/6j mice (36). The training speed was increased by 1 m/min every week, and at the end of the study, the mice ran until exhaustion. After the tests the mice were sacrificed, and the tissue samples were carefully dissected, snap-frozen in liquid nitrogen and stored at −80°C until RNA or proteins were extracted. The COX10-KO, Pgc1α overexpressors and [COX10-KO × Pgc1α] mice have been previously described (18,23).

RNA extraction

Total cellular RNA was extracted from frozen tissue samples in TRIzol reagent (Invitrogen) and homogenized with a Dounce homogenizer with 30 consecutive strokes. Subsequent steps were performed according to the manufacturer’s instructions. A Qiagen RNeasy Mini Kit was used to extract the total RNA after the TRizol protocol. The quality of RNA was tested by analyzing the intensities and ratios of cytoplasmic 18S and 28S rRNAs with a 2100 Bioanalyzer (Agilent Technologies).

Gene expression profiling

Gene expression profiles were determined from quadriceps femoris of three female Deletor and four female control littermate mice, aged 21–24 months, using the GeneChip Mouse Genome 430 2.0 array (Affymetrix). One microarray experiment contained RNA from a single mouse. Labeling, hybridization and scanning were performed according to manufacturer’s instructions. Data were normalized according to the Robust Multichip Average procedure, RMA (37), using the Genespring 7.2 software. In order to determine those genes that were differentially expressed between transgenic and control mice, a t-test was applied. The transcripts that fulfilled the P-value criterion of <0.05 for differential expression between transgenic and control mice were selected for further analysis regardless of fold change. The false-positive rate (FDR) was determined as in Zapala et al. (38) to be 0.67%. The selected transcripts were subjected to pathway analysis using the Ingenuity Pathway Analysis (Ingenuity Systems), a literature mining curated database.

Bioinformatic promoter analysis

Promoter regions were predicted with Genomatix software, and analyzed using the ModellInspector tool of Genomatix (http://www.genomatix.de/). Module library 5.3 and vertebrate modules were used in the model search.

Reverse transcription–PCR and quantitative PCR

One microgram of total RNA was used to generate cDNA using random hexamers with Moloney murine leukemia virus reverse transcriptase (Promega). The qRT-PCRs were performed on cDNA with a DyNaMo Flash SYBR Green qRT-PCR Kit (Finnzymes) on an ABI prism 7000 light cycler. Gapdh, β2-microglobulin and β-actin were used as control genes. The primer sequences are available on request.
Histochemistry

The mouse *quadriceps femoris* muscle samples were fixed in 10% buffered formalin and embedded in paraffin. The primary antibodies used were monoclonal anti-CII-Fp (MS204, Molecular Probes, working dilution 1:200) and goat anti-mouse FGF21 antibody (AF3057, R&D Systems, 1:1000). The samples were blocked for non-specific staining by incubating for 30 min in 2% horse or rabbit serum, respectively. Further detection was carried out with the Vectastain ABC Mouse or Goat IgG Kits (Vector Laboratories) according to the manufacturer’s instructions followed by chromogen DAB staining. The slides were briefly counterstained with hematoxylin. Light microscopy was performed with Axioplan 2 (Carl Zeiss).

For the adipocyte size quantification, formalin-fixed paraffin sections were H&E stained and studied under an Axioplan 2 microscope. The size of adipocytes (on average 185 ± 87 cells per mouse) was quantified with the ImageJ program (http://rsbweb.nih.gov/ij/index.html).

The Oil Red O staining was performed on 8 µm frozen tissue sections from liver. The slides were fixed in formic calcium (1:10:1 concentrated formalin:aqua:10% calcium chloride) for 5 min. Slides were then incubated in Oil Red O solution for 10 min and the nuclei were stained with Mayer’s hematoxylin for 2 min. Oil Red O stock solution (1 mg/ml in isopropanol) was made by heating the reagent and alcohol at 56°C for 30 min. Fresh working solution was made from stock solution and aqua (3:2). The solution was mixed well, let to stand for 5 min, filtrated and used within a few hours. The ATPase staining was done according to routine histological protocols, in preincubation pH 4.3, 4.6 and 10.4.

Quantitative western blot analysis

The proteins from muscle samples were extracted by homogenizing in RIPA buffer (0.15 M NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.05 M Tris–HCl, pH 8.0, 2 mM DTT, protease inhibitors). The samples were incubated on ice for 20 min and centrifuged at 13 000 g for 5 min. The supernatant was collected and stored at −80°C. Protein samples were separated on 15% SDS–PAGE gels and transferred to an Immobilon-FL transfer membrane (Millipore) using the Bio-Rad Western Transfer unit. The membranes were blocked in 1% bovine serum albumin (BSA; for Fgf21) or 5% milk (for Mthfd2 and Actin), or 5% BSA for all other antibodies in TBS–TWEEN 20 (0.1%) for 1 h at room temperature. Blocking solutions were replaced with fresh solution and antisera at the appropriate dilution. The primary antibodies used were goat anti-mouse Fgf21 antibody (AF3057, R&D Systems, 1:10 000) chicken polyclonal Mthfd2 (ab37840, Abcam), goat polyclonal Actin (sc-1616, Santa Cruz Biotechnology, 1:1000) and 1:1000 also for rabbit polyclonal anti-Akt total (4691, Cell Signaling), anti-chicken IgG (Abcam), goat polyclonal Actin (sc-1616, Santa Cruz Biotechnology, 1:1000) and 1:1000 also for rabbit polyclonal antibodies pan Akt total (4691, Cell Signaling), pAkt S473 (4060, Cell Signaling) or anti-chicken IgG (Abcam) was incubated with the membranes at a dilution of 1:10 000 in blocking buffer at room temperature for 1 h. The secondary antibody was removed and filters washed three times in TBS ± 0.1% Tween 20 for a total of 15 min. An ECL Plus Western Blotting Detection System (GE Healthcare) was used for the detection of signals with Typhoon 9400 (Amersham Biosciences) and quantified with the ImageQuant v5.0 software.

Metabolic measurements

The plasma and serum samples were prepared by centrifugation after blood collection and stored at −80°C until analyzed. Mouse plasma Fgf21 concentrations were measured using a radioimmunoassay kit (Phoenix Pharmaceuticals, Inc.). Serum growth hormone and insulin concentrations were determined using an ELISA kit for mouse/rat (LINCO Research, Millipore). Serum T3 concentrations were determined using an ELISA kit for mouse/rat (Calbiotech) and TSH using an ELISA kit for mouse/rat (Gentaur). For the insulin tolerance test, mice were fasted 6 h and challenged by i.p. insulin injection of 1 U/kg (NovoRapid, Novo Nordisk A/S). Blood samples were taken from the tail vein and blood glucose levels were measured using a Precision Xceed glucometer (Abbot Laboratories) immediately prior to insulin administration, and at 30, 60 and 90 min after insulin administration. For the oral glucose tolerance test, mice were fasted 6 h and challenged by an oral glucose load (2 g/kg). Blood glucose levels were measured prior to glucose administration, and at 15, 30 and 90 min after glucose administration.

Metabolomic analysis

The metabolomic analysis was done using the GCxGC-TOF/ MS platform for amino acid measurements, exactly as in Ahola-Erkki et al. (14).

Statistical analysis

The statistical analyses were performed using Graph Pad Prism 5. Student’s *t*-test was used in all comparisons of mouse data. All error bars represent the standard error of the mean.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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