Effect of bezafibrate treatment on late-onset mitochondrial myopathy in mice

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Mitochondrial dysfunction is an important cause of metabolic disorders of children and adults, with no effective therapy options. Recently, induction of mitochondrial biogenesis, by transgenic overexpression of PGC1-alpha [peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1-alpha], was reported to delay progression of early-onset cytochrome-c-oxidase (COX) deficiency in skeletal muscle of two mouse models: a muscle-specific knock-out of COX10 (COX10-mKO) and a constitutive knock-out of Surf1 (Surf1-KO). A pan-PPAR agonist, bezafibrate, could similarly delay myopathy progression in COX10-mKOs, but not in SURF1-KOs. We asked whether bezafibrate affected disease progression in late-onset adult-type mitochondrial myopathy mice. These ‘Deletor mice’ express a dominant patient mutation in Twinkle-helicase, leading to accumulation of multiple mtDNA deletions and subsequent progressive respiratory chain (RC) deficiency with COX-negative muscle fibers at 12 months of age. The primary and secondary molecular findings in Deletor mice mimic closely those in patients with Twinkle myopathy. We applied 0.5% bezafibrate diet to Deletors for 22 weeks, starting at disease manifestation, mimicking patient treatment after diagnosis. Bezafibrate delayed significantly the accumulation of COX-negative fibers and multiple mtDNA deletions. However, mitochondrial biogenesis was not induced: mitochondrial DNA copy number, transcript and RC protein amounts decreased in both Deletors and wild-type mice. Furthermore, bezafibrate induced severe lipid oxidation effects, with hepatomegaly and loss of adipose tissue, the mechanism involving lipid mobilization by high hepatic expression of FGF21 cytokine. However, as bezafibrate has been tolerated well by humans, the beneficial muscle findings in Deletor mice support consideration of bezafibrate trials on adult patients with mitochondrial myopathy.

INTRODUCTION

Mitochondrial disorders are an important cause of metabolic disorders in patients of any age. A majority of these disorders affect the respiratory chain (RC) and the cellular ATP production, but even these clinical manifestations are highly variable, extending from early childhood encephalopathies to adult-onset myopathies. In spite of often rapidly progressive, devastating disease course, no curative therapy options are available and even palliative therapy tools are scarce (1). Therapy trials have been hampered by heterogeneity of disease manifestations in humans, rendering homogeneous study groups small. However, recent development of animal models has offered a new tool to test therapeutic strategies (2).

Genetically modified mice as mitochondrial dysfunction models are created with different strategies and manifest with a wide range of phenotypes (2). When these mice are used for therapy trials, it is important to consider what kind of mitochondrial disorders the findings are relevant for, when drawing conclusions of potential efficacy of the intervention and extrapolating the findings to humans. If an essential mitochondrial protein affecting, e.g. the RC is ‘knocked out’, the result in mice is typically early fetal lethality (2). Therefore, tissue-specific conditional knock-outs are often utilized. Mice with muscle-specific inactivation of COX10 (COX10-mKO), an assembly factor of COX (complex IV of RC), developed progressive severe COX deficiency and mitochondrial myopathy, leading to the death of the mice at ~4

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months of age (3). The closest human disease phenotype that mimics these muscle-targeted COX10 mice is infantile fatal COX deficiency (4,5), whereas for humans COX10 mutations cause infantile encephalopathy or cardiomyopathy (6). Mutations in Surf1, also involved in COX assembly, lead in humans to isolated severe COX deficiency and early-childhood mitochondrial encephalopathy, Leigh disease (7). However, when knocked out constitutively in mouse, the lack of Surf1 causes no apparent phenotype, but COX shows 30–40% residual activity compared with wild-type in all analyzed tissues, also visualized by COX-negative, mostly type-2, muscle fibers (8). Therefore, Surf1-KO mice are a model for modest overall COX-activity reduction, with no direct disease equivalent. A third example of mouse models for mitochondrial disease is the Deleter mouse, which overexpresses a dominant patient mutation in mitochondrial replicative helicase Twinkle, leading to accumulation of multiple mtDNA deletions and progressive RC deficiency (9). At 12 months of age, COX-negative fibers start to appear, increasing in number by age. The type of manifestation and molecular findings mimic closely the human disorder, autosomal dominant mitochondrial myopathy (10), and some secondary physiological consequences of the disorder in mouse muscle (11) have been replicated in human patients with different mitochondrial disorders (12), supporting that Deletors are an optimal animal model for adult-onset mitochondrial myopathies.

Based on studies with COX10-mKO, Surf1-KO mice and Deleter mice, mitochondrial biogenesis induction has been suggested to be a potential therapeutic strategy for mitochondrial disease (13–15). Ketogenic diet stimulated mitochondrial oxidative metabolism in Deletors, reducing the amount of COX-negative fibers (15). Another way to affect mitochondrial biogenesis is to induce master regulators of mitochondrial metabolism: peroxisome proliferator-activated receptors (PPARs) are fatty-acid-regulated nuclear receptors, with three identified isoforms, α, β/δ and γ (16–19), and PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1-alpha) is a transcription co-activator, known to interact with many nuclear receptors also outside PPARs (20–22) and to be essential for mitochondrial biogenesis, along with its homologue β (23). PPARs and PGC-1α regulate e.g. fatty acid storage or utilization by beta oxidation, thermogenesis, muscle metabolism and fiber type determination (24–26). When COX10-mKO and Surf1-KO mice were crossed with mice overexpressing PGC-1α, COX activity was partially rescued (13,15). In COX10m-KO mice, lethality was not prevented, but the lifespan of the animals was increased by five additional months (13). When COX10-mKO mice were given bezafibrate, a pan-PPAR-agonist typically used to treat human hyperlipidemia, COX activity was also improved, mimicking the effect of PGC-1α overexpression (13). However, bezafibrate did not affect COX activity in Surf-KO mice (15). Based on these studies, induction of mitochondrial biogenesis and fatty-acid metabolism may be beneficial for different kinds of RC deficiency models, but discrepancies still exist between different models.

We report here the effect of bezafibrate in an adult-onset mitochondrial myopathy model, the Deleter mouse. Bezafibrate treatment was started at the time of disease manifestation, mimicking the situation of patients at diagnosis. Our results suggest that therapy trials with bezafibrate-like drugs in adult patients with mitochondrial myopathy are warranted.

RESULTS

Bezafibrate reduced the amount of COX-negative fibers and mtDNA deletion load in Deleter mice

We administered bezafibrate diet (BD) and control diet (CD) to the Deleter and WT mice starting at 12 months of age, at the time of disease onset, i.e. when COX-negative fibers start to appear. These mice were sacrificed at 17.5 months of age, and the muscle phenotype was scored. Bezafibrate decreased the amount of COX-negative fibers moderately in both genders: the males in BD had 1.5 times less and females two times less COX-negative fibers than those in CD (Fig. 1A–C). No apparent induction of COX activity was visible after BD in any mice, and no COX-negative fibers were found in WT mice at 17.5 months of age. mtDNA deletion load was decreased in both Deleter males (P = 0.0447) and females (P = 0.0230) after BD (Fig. 1D).

Treadmill tests were performed every 3 weeks, but no significant difference was seen between bezafibrate-fed and normal diet-fed mice (data not shown).

Bezafibrate suppressed mitochondrial biogenesis in Deleter mice

Bezafibrate has been previously reported to induce mitochondrial biogenesis in COX10-mKO mice (13). However, in Deletors it did not have a similar effect. Citrate synthase (CS) activity, a marker for mitochondrial mass, was unaffected or decreased by BD (Fig. 1E). mtDNA copy number was decreased after BD in all samples, from 47–59% in Deletors to 49–71% of the level of wild-type mice in CD. To study whether reduced mtDNA copy number affected mtDNA transcript levels, we studied the level of transcripts encoding of ND2 (complex I subunit), COX I (complex IV subunit) and cyt b (complex III gene encoding component), and normalized their expression to that of nuclear gene β-actin (Fig. 1G–I). Complex I was moderately decreased or unchanged also in the protein level after BD (Fig. 1J), and complex IV was decreased or unchanged after BD in all mice (Fig. 1J). These results indicate that bezafibrate down-regulated mitochondrial DNA maintenance and transcription, as well as mitochondrial biogenesis in Deleter mice.

Significant loss of body weight, reduction of adipose tissue and decrease of body temperature in BD mice

Bezafibrate resulted in severe loss of body weight in all male and female mice (Fig. 2E). Accordingly, adipose tissue volume and adipocyte size also decreased significantly (Fig. 2A–C). When the respiratory quotient (RQ) of the mice was studied, the BD-fed mice showed higher CO2 elimination and O2 consumption (Fig. 2D), which was associated with a tendency to lower body temperature of BD males.
The severely reduced body weight and adipose tissue insulation likely required increased heat generation to maintain body temperature, partially explaining the changes in the RQ.

Figure 1. Effect of BD on Deletor mitochondrial myopathy. Deletor male mice at 17.5 months of age on BD (A and C) showed less COX-negative fibers than those in CD (B and C) (5.1 ± 0.5% versus 7.7 ± 0.4%, respectively; P = 0.0011; 1000–2000 muscle fibers of QF counted per mouse, n = 7–8 mice per group), and females on BD (C) had less COX-negative fibers than those on CD (1.36 ± 0.13% versus 2.36 ± 0.28, respectively; P = 0.0064; n = 6–7 mice/group, 1000–2000 fibers counted). Arrows indicate examples of COX-negative fibers in COX/SDH staining. (D) Effect of BD on mtDNA deletion load (n = 7). (E) Effect of BD on CS activity, as a marker of mitochondrial mass (n = 6–9). (F) Effect of BD on mtDNA copy number (n = 6–9). (G–I) Quantification of ND2, Cyt b and COX I mRNA levels after BD or CD (n = 6–9). (J) Representative western blot (SDS–PAGE) analysis of RC enzymes, showing the NDUFA9 subunit of complex I, and COX4 subunit of complex IV in skeletal muscle of mice on either BD or CD (complex I, n = 4–7 and complex IV, n = 2–7). Scale bar in (A) and (B): 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001.

Hepatomegaly and hepatic lipid oxidation induced by bezafibrate

Bezafibrate caused considerable hepatomegaly, increasing the liver/body weight ratio to almost double in all mouse groups.
Figure 2. Adipose tissue and metabolic phenotype of mice after BD. (A and B) BD induced small size of adipocytes ($n = 6–9$). (C) Abdominal fat was decreased in BD mice. (D) $O_2$ consumption and $CO_2$ elimination, when recorded for 48 h repeatedly during BD and CD (black line indicates dark period, times indicate daily times at recording) ($n = 4–7$). (E) Body weight of Deleter and WT mice upon BD ($n = 6–9$). (F) Body temperature in Deleter and WT male mice after BD ($n = 4–7$). Scale bar in (A): 50 $\mu$m, and in (C): 1 cm. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 
In spite of moderate increase of fat in the liver [Fig. 3B; male shown; hematoxylin–eosin (HE) on paraffin sections, showing fat as empty white droplets; Oil red O lipid staining on frozen sections, showing fat as red droplets], most of the size increase was caused by hepatoproliferative effect of bezafibrate. Uncoupling protein-2 mRNA expression level in BD mice was markedly elevated compared with each corresponding CD mouse group (Fig. 3C). Transcripts for proteins involved in lipid oxidation, such as fatty-acid translocase CD36 and peroxisomal acyl-coA oxidase 1, ACOX1, were elevated significantly in BD mice compared with CD mice, indicating induction of lipid oxidation upon BD (Fig. 3D and E). Although the liver size was almost doubled, bezafibrate resulted in mtDNA copy number decrease (Fig. 3F).

These results show that bezafibrate had a major effect on hepatic metabolism in all mice, with induction of lipid oxidation and mitochondrial uncoupling, as well as hepatocyte proliferation.

PPARs and PGC-1α mRNA expression did not change after BD

In skeletal muscle or liver, PPAR-α, -δ and -γ mRNA or PGC-1α expression levels were not increased after BD of 22 weeks (Supplementary Material, Fig. S1). Hexokinase (HK) 2 as a marker of glycolysis was unchanged in skeletal muscle (Fig. 4B), as well as ACOX1 and CD36 (Fig. 4C).
and D). These results indicate that bezafibrate did not chronically increase PPARs or PGC-1α, or lipid oxidation in muscle.

Liver induces FGF21 secretion upon bezafibrate treatment

Because of extensive lipolysis upon bezafibrate treatment, we analyzed the plasma concentration for FGF21, a cytokine known to induce lipolysis in adipose tissue (11,27). FGF21 concentrations were increased up to 10-fold in BD mice compared with CD mice (Fig. 3H), which may explain the loss of adipose tissue in the mice. FGF21 mRNA expression level in the liver was significantly increased in BD mice compared with CD mice (Fig. 3G), whereas skeletal muscle FGF21 expression was not (Fig. 4A), suggesting that serum FGF21 was of hepatic origin. We have previously described induction of muscle FGF21 in the muscle of Deletor mice, and correlation of FGF21 expression to the amount of COX-negative fibers (11). Here, along with the reduction of COX-negative fibers, also FGF21 expression slightly reduced in Deletor muscle upon BD.

DISCUSSION

We report here that bezafibrate delayed the progression of late-onset adult-type mitochondrial myopathy, i.e. development of COX-negative fibers and multiple mtDNA deletions, in Deletor mice. Previously, in a model of early-onset severe COX deficiency (COX10-mKO mice), bezafibrate corrected COX activity and induced mitochondrial biogenesis (13). However, in a second report on an early-onset partial COX-deficiency model (Surf1-KO mice), no effect was seen on muscle RC activity, but the treatment was accompanied by severe lipid metabolic side effects and hepatomegaly (14). Our results in a late-onset disease model, closely mimicking the histological and physiological changes of human adult patients with mitochondrial myopathy (11,12), suggest that bezafibrate could delay disease progression in RC deficiency of skeletal muscle in adults, but that in mice beneficial effects are overridden by severe hepatic side effects, which have not, however, been reported in humans (28).

In the Deletor mice, the improved muscle condition after BD was not explained by chronic mitochondrial biogenesis induction, as all the inducers or signs of mitochondrial biogenesis, including the master regulators of mitochondrial biogenesis (PGC-1α, PPAR-α, -δ and -γ), mtDNA copy number, mtDNA transcripts, RC protein amounts and CS activity, were either unchanged or down-regulated in tissue homogenates. However, we cannot exclude the possible induction of these proteins at early-stage diet. Furthermore, as only a fraction of muscle fibers manifest the COX defect, and as we analyzed muscle homogenates, subtle changes in the defective fibers could be diluted out by neighboring COX-normal muscle fibers. A cause of the overall decrease in mtDNA could be a response to chronically increased beta-oxidation, possibly interpreted by the muscle as high oxidative ATP production. Studies of bezafibrate effects in different mitochondrial mouse models are partially conflicting, as PGC-1α has been reported to be increased (13) or normal (14) and PPAR-α, -δ and -γ increased (13,14), or normal (this study). The effect of bezafibrate on PPARs was reported to be dose-dependent (29), a high-dose bezafibrate (100 mg/kg/d;
gavage administration) inducing hepatic PPAR-α expression, decreasing PPAR-β and having no effect on PPAR-γ expression. The variation in the mitochondrial disease models cannot, however, be explained by varying dosage, as all studies used the same high-dose diet scheme, 0.5% added on diet, equaling to 300–500 mg/kg/d, that is 45–75-fold higher than typically used for humans, warranting caution in result extrapolation to humans. However, considerable similarities between the findings in the three studies were also evident, i.e. severe weight loss (this study and 14), effect on COX-negative fibers (this study and 13) and lipid oxidation induction and hepatomegaly (this study and 14). These findings indicate that these effects are related to bezafibrate effect on the tissues, and are not explained by induction of PPARs or mitochondrial biogenesis.

We found that bezafibrate significantly induced the expression of FGF21, which is a starvation-induced hormone, secreted in fasting to blood stream from the liver, and acting on adipose tissue to mobilize lipids (30). Elevated FGF21 has been reported to activate fatty acid oxidation and inactive lipid synthesis via PGC-1α in liver (31), but also PGC-1α was found to negatively regulate hepatic FGF21 mRNA expression and plasma level (32). In our mice, FGF21 induction was accompanied by enhanced mRNA expression of genes involved in hepatic fatty acid uptake and lipid oxidation, without increased expression of PPARs and PGC-1α, agreeing with a previous suggestion of direct bezafibrate action on FGF21 in the liver (33). FGF21 is known to release fat from adipose tissue and liver and to induce beta oxidation (27). Therefore, the bezafibrate-induced severe weight loss, absence of visceral fat and induction of lipid oxidation in the mitochondrial mouse models may be mediated by this hormone.

The progressive metabolic phenotype of our mice on bezafibrate was demonstrated by progressively increasing O₂ consumption during the diet. Increased O₂ consumption was accompanied by induction of UCP2 expression. High O₂ consumption has previously been linked with BD in mice (34) as well as UCP2 induction in the rat liver (35). High O₂ consumption was likely to be partially due to maintenance of body temperature upon loss of adipose tissue insulation. However, our results also are consistent with bezafibrate-induced UCP2-mediated mitochondrial proton leak in the liver, leading to increased O₂ consumption and a hypermetabolic state.

In conclusion, bezafibrate resulted in milder progression of adult-onset myopathy in Deletor mice, slowing down accumulation of mtDNA deletions and COX-negative fibers. However, these positive effects were overridden by extensive effects of the drug on the liver, leading to major effects on lipid utilization and a hypermetabolic state, as well as hepatoproliferative syndrome, doubling the size of the liver. These liver effects are likely rodent-specific, and bezafibrate has been tolerated well by humans. As FGF21 levels in this study were indicative of hepatoproliferative syndrome and the same marker is indicating RC deficiency in the muscle of mice and men (11,12), blood concentration of FGF21 could be used to follow up both efficacy and side effects of the drug: decreasing levels could indicate both the absence of liver hypermetabolism and a positive effect on RC deficiency in the patient’s muscle. Our findings of bezafibrate effect on respiratory-chain-deficient skeletal muscle may support consideration of this drug for trials on adult patients with mitochondrial myopathy.

**MATERIALS AND METHODS**

**Animals**

All animal procedures were performed according to protocols approved by the ethical boards for animal experimentation of Helsinki University as well as State Provincial offices of Finland, and all experiments were performed in accordance with good practice of handling laboratory animals and of genetically modified organisms. This study used a previously described mouse model, the Deletor mouse (9). Deletor mice were congenic C57BL/6. This study consisted of 31 Deletor mice (16 males) and 31 WT mice (14 males). The mice were bred to one to five per cage in a room under controlled temperature at 21°C with 12 h light—12 h dark cycle (lights on at 6:00 and lights off at 18:00), and ~60% humidity. The mice had similar life span and body weight compared with WT mice. Their quadriceps femoris (QF) showed: (i) No COX-negative fibers until 12 months of age in males and females; (ii) gradual increase of COX-negative fibers from 12 months of age, the proportion of which in QF at 18–24 months of age was 11.8 ± 4.4%; (iii) in Deletor males, three times more COX-negative muscle fibers than Deletor females at 14 months of age (5.6 ± 0.9 versus 1.9 ± 0.2%, respectively; P < 0.0001) (15). Based on these data, we selected the starting age of bezafibrate treatment to be upon disease manifestation, at 12 months of age. CD and BD were administered to the Deletor and WT mice at 12 months of age, and these mice were sacrificed at 17.5 months of age.

The Deletor and WT mice of 12 months of age were fed pellets of CD (D12450B, rodent diet with 10% fat, Research Diets, Inc., New Brunswick, NJ, USA) or BD (D12450B with 0.5% bezafibrate, Research Diets, Inc.). Both diets consisted of the same amount of calories. The mice were divided into eight groups: CD was fed to Deletor male (n = 8), Deletor female (n = 7), WT male (n = 6) and WT female (n = 9); BD was fed to Deletor male (n = 8), Deletor female (n = 8), WT male (n = 8) and WT female (n = 8). The mice had free access to water and pellet. The mice were sacrificed by carbon monoxide gas, and blood, QF, visceral fat and liver were collected afterward. Whole liver was removed and weight was measured.

Body weight was measured every 3 weeks, and simultaneously treadmill exercise test (Exer-6M Treadmill, Columbus Instrument) was performed with an initial speed of 7 m/min for 2 min, which was increased by 2 m/min after every 2 min. The test was discontinued after the mice fell from the belt on the stimulus site for 10 s continuously, or fell 10 times.

The RQ study constituted of seven Deletor male mice and four WT male mice. The mice were kept in single mouse per cage, and otherwise all conditions were similar to the main study. The study was conducted at 12 months of age.
Morphologic analysis

We collected samples from QF and liver. QF and liver sections were embedded with OCT Compound Embedding Medium (Tissue-Tek®) and frozen in 2-methylbutane in liquid nitrogen. Frozen QF sections (12 μm) were stained with COX and SDH simultaneously. Frozen liver sections (8 μm) were stained with Oil Red O, which were performed as previously described (15). COX-SDH-stained QF was analyzed for abnormal muscle fibers. We chose the three most severely affected fields in a section on ×200 magnification (Axioplan 2 Universal Microscope, Zeiss), captured, and then counted the COX-negative, COX-negative and SDH-positive and normal fibers. We counted totally approximately 1000–2000 muscle fibers from each QF.

mtDNA quantification

Total DNA was extracted from frozen QF by standard proteinase K and phenol–chloroform methods. We measured mtDNA copy number by quantitative PCR (qPCR). Twenty-five nanograms of total DNA was used to generate cDNA using random hexamers (Random Primer, #C1181, Promega), ribonuclease inhibitor (Recombinant RNasin, #N2511, Promega) with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV reverse Transcriptase, #M1701, Promega). Quantitative real-time PCRs were performed using cDNA generated from mouse QF and liver with DyNAmo Flash SYBR Green qPCR Kit (Finzymes) on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Amplification conditions were: 95°C for 7 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Quantification was confirmed to be specific and exponential. Primer sequences used in this study were as follows: ND2 (forward 5′-AGGGATCCCACCTGCACATG-3′ and reverse 5′-CTCCTCACTGCCCTATGAAA-3′), Cytb (forward 5′-GC TTCCACTTCATCTTTACATTTA-3′ and reverse 5′-TGTT GGTTGGTGTGATCTCG-3′), COX I (forward 5′-TGGAG CCCTTTGAAACCTGAC-3′ and reverse 5′-TCCTGCACTG GAGTTC-3′), PPAR-α (forward 5′-CCTCCAGGTACCCA TACAGGAGT-3′ and reverse 5′-GCCGTAATGTCCGCG AA-3′), PPAR-δ (forward 5′-TCAACATGGAAATGTCG GTGTG-3′ and reverse 5′-ATACTCTGAGCTTCATGGCGATT- 3′), PPAR-γ (forward 5′-CCTGCGCAAGAGCTCTTGAT-3′ and reverse 5′-AAATCCCTGGCCCTCTGAGAT-3′), PGC-1α (forward 5′-CGGAACATATCATCAACACG-3′ and reverse 5′-TGAGGACCCTAGCAAGTTTG-3′), FGF21 (forward 5′-CTGGGTTGTGCTACCAAGCAT-3′ and reverse 5′-CACC CAGATTGTAATGACC-3′), UCP2 (forward 5′-TCTGGA TACCCGCAAGGT-3′ and reverse 5′-TGGTAGAGGG TTGCTGAGA-3′), HK2 (forward 5′-CTGTCTACAGAAC ATCCCCATT-3′ and reverse 5′-CACCACCGCTACCACCAT G-3′), ACOX (forward 5′-ACCGCTATGCTTCCATTTTC-3′ and reverse 5′-GCCGAGCTCCAGGACAT-3′ and reverse 5′-GCAAGAATCGCCACCTTTG-3′), CD36 (forward 5′-CCAATAGAAGTGACAGCATAGGACAT-3′ and reverse 5′-GTGGACCTGAGTTCCTTTCC-3′) and β-actin (forward 5′-ATGTTCCTCCGGGCTGTAT-3′ and reverse 5′-CATTAGGAGTCCTTCTGACCCATT-3′).

Western blotting

Total cell lysates were extracted from a piece of QF in RIPA buffer with protein inhibitor (Complete Mini, Roche) and homogenized on ice. Protein concentration was measured using the Bradford method (Protein Assay, Bio-Rad). Ten micrograms of total protein was used to generate cDNA using random hexamers (Random Primer, #C1181, Promega), ribonuclease inhibitor (Recombinant RNasin, #N2511, Promega) with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV reverse Transcriptase, #M1701, Promega). Quantitative real-time PCRs were performed using cDNA generated from mouse QF and liver with DyNAmo Flash SYBR Green qPCR Kit (Finzymes) on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Amplification conditions were: 95°C for 7 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Quantification was confirmed to be specific and exponential. Primer sequences used in this study were as follows: ND2 (forward 5′-AGGGATCCCACCTGCACATG-3′ and reverse 5′-CTCCTCACTGCCCTATGAAA-3′), Cytb (forward 5′-GC TTCCACTTCATCTTTACATTTA-3′ and reverse 5′-TGTT GGTTGGTGTGATCTCG-3′), COX I (forward 5′-TGGAG CCCTTTGAAACCTGAC-3′ and reverse 5′-TCCTGCACTG GAGTTC-3′), PPAR-α (forward 5′-CCTCCAGGTACCCA TACAGGAGT-3′ and reverse 5′-GCCGTAATGTCCGCG AA-3′), PPAR-δ (forward 5′-TCAACATGGAAATGTCG GTGTG-3′ and reverse 5′-ATACTCTGAGCTTCATGGCGATT- 3′), PPAR-γ (forward 5′-CCTGCGCAAGAGCTCTTGAT-3′ and reverse 5′-AAATCCCTGGCCCTCTGAGAT-3′), PGC-1α (forward 5′-CGGAACATATCATCAACACG-3′ and reverse 5′-TGAGGACCCTAGCAAGTTTG-3′), FGF21 (forward 5′-CTGGGTTGTGCTACCAAGCAT-3′ and reverse 5′-CACC CAGATTGTAATGACC-3′), UCP2 (forward 5′-TCTGGA TACCCGCAAGGT-3′ and reverse 5′-TGGTAGAGGG TTGCTGAGA-3′), HK2 (forward 5′-CTGTCTACAGAAC ATCCCCATT-3′ and reverse 5′-CACCACCGCTACCACCAT G-3′), ACOX (forward 5′-ACCGCTATGCTTCCATTTTC-3′ and reverse 5′-GCCGAGCTCCAGGACAT-3′ and reverse 5′-GCAAGAATCGCCACCTTTG-3′), CD36 (forward 5′-CCAATAGAAGTGACAGCATAGGACAT-3′ and reverse 5′-GTGGACCTGAGTTCCTTTCC-3′) and β-actin (forward 5′-ATGTTCCTCCGGGCTGTAT-3′ and reverse 5′-CATTAGGAGTCCTTCTGACCCATT-3′).
RQ analysis

We measured RQ using Oxymax Lab Animal Monitoring System (CLAMS; Columbus Instruments, OH, USA). The RQ study required 3 days; first day and night is preparation time for mice to adjust to the new conditions, and on the second and third days, CO₂ elimination and O₂ consumption were measured. Body weight and rectal body temperature were measured on the first day. The mice were fed powder diet, which was crushed by hand from pellet BD due to the cage feeding system of CLAMS.

Adipose tissue analysis

Adipose tissue was extracted from the abdomen. Adipose tissue was fixed by 4% formalin, sections (8 μm) were cut and stained by HE. We chose the three random fields in the sections on ×200 magnification (Axioplan 2 Universal Microscope, Zeiss), for measuring adipocyte dimensions. Adipocyte area was measured using the public domain software ImageJ (NIH).

Statistical analysis

All figures except for CS activity were expressed as means ± SEM. One-way ANOVA was used to test for body weight in the main study, and body weight and body temperature measurement in the RQ study, and Tukey’s test was performed as a post hoc test. Other studies were evaluated using unpaired t-test alone or combined with a Welch correction when variances were significantly different. Statistical analyses were performed with PRISM 5.0 for Mac OS X (GraphPad software).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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