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Impaired fatty acid oxidation in a *Drosophila* model of mitochondrial trifunctional protein (MTP) deficiency

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ABSTRACT

Mitochondrial trifunctional protein (MTP), which consists of the MTPα and MTPβ subunits, catalyzes long-chain fatty acid β-oxidation. MTP deficiency in humans results in Reye-like syndrome. Here, we generated *Drosophila* models of MTP deficiency by targeting two genes encoding *Drosophila* homologs of human MTPα and MTPβ, respectively. Both $Mtp\alpha^{KO}$ and $Mtp\beta^{KO}$ flies were viable, but demonstrated reduced lifespan, defective locomotor activity, and reduced fecundity represented by the number of eggs laid by the females. The phenotypes of $Mtp\alpha^{KO}$ flies were generally more striking than those of $Mtp\beta^{KO}$ flies. $Mtp\alpha^{KO}$ flies were hypersensitive to fasting, and retained lipid droplets in their fat body cells as in non-fasting conditions. The amount of triglyceride was also unchanged upon fasting in $Mtp\alpha^{KO}$ flies, suggesting that lipid mobilization was disrupted. Finally, we showed that both $Mtp\alpha^{KO}$ and $Mtp\beta^{KO}$ flies accumulated acylcarnitine and hydroxyacylcarnitine, diagnostic markers of MTP deficiencies in humans. Our results indicated that both $Mtp\alpha^{KO}$ and $Mtp\beta^{KO}$ flies were impaired in long-chain fatty acid β-oxidation. These flies should be useful as a model system to investigate the molecular pathogenesis of MTP deficiency.

1. Introduction

Mitochondrial β-oxidation is the major metabolic system to catabolize fatty acids, yielding the primary energy source for various cellular processes. Beta-oxidation produces acetyl-CoA by degrading fatty acids via a cascade of four reactions: dehydration, hydration, oxidation, and thiolysis. Mitochondrial trifunctional protein (MTP) catalyzes the latter three steps in the β-oxidation of long-chain fatty acids [1–3]. MTP consists of a hetero-octamer of four MTPα and four MTPβ subunits. The MTPα subunit possesses long-chain 3-enoyl-CoA hydratase and long-chain 3-hydroxyacyl-CoA dehydrogenase activities, which catalyze the second and third steps, respectively. The MTPβ subunit has long-chain 3-ketoacyl-CoA thiolase activity, which catalyzes the fourth step.

Mutations in either MTPα or MTPβ are thought to cause reduced activities of all three enzymes [4,5]. MTP-deficient patients exhibit feeding difficulties, low blood sugar, muscle weakness, Reye-like syndrome, and sudden death in infancy [6–10]. Animal models of MTP deficiency have been reported in mice: *Mtpα* knockout ($Mtp\alpha^{KO}$) mice developed hepatic steatosis after birth, and exhibited neonatal hypoglycemia and sudden death; mice bearing a point mutation in *Mtpβ* was viable, but show a decreased weight gain, hepatic steatosis, and cardiomyopathy [11,12]. All MTP-deficient mammals exhibit accumulation of long-chain fatty acid metabolites, indicating that MTP plays crucial roles in long-chain fatty acid oxidation in vivo.

The fruit fly *Drosophila melanogaster* shares many genes with humans—approximately 75% of disease-related genes in humans have functional orthologs in the fly [13]—and it has been used as a model system to study the molecular and genetic bases of human disease. The models can be generated by making either loss-of-function mutants or transgenic flies bearing human disease genes [14–16]. In the present study, we generated *Drosophila* models of MTP deficiency using the gene-targeting method. Both $Mtp\alpha^{KO}$ and $Mtp\beta^{KO}$ flies were viable, but had clear phenotypes, which include a shortened lifespan, defective locomotor activity, reduced fecundity, and abnormal lipid catabolism, which was enhanced when animals were fasted. The phenotypes were generally more striking in $Mtp\alpha^{KO}$ than in $Mtp\beta^{KO}$ flies. We demonstrated that both $Mtp\alpha^{KO}$ and $Mtp\beta^{KO}$ flies accumulate acylcarnitine and hydroxyacylcarnitine, the intermediates of long-chain fatty acid metabolism, indicating that they have defects in long-chain fatty acid oxidation. These results suggest that the function of MTP is conserved between mammals and *Drosophila*, and that the *Drosophila* model should be useful to understand the molecular pathophysiology of MTP deficiency.

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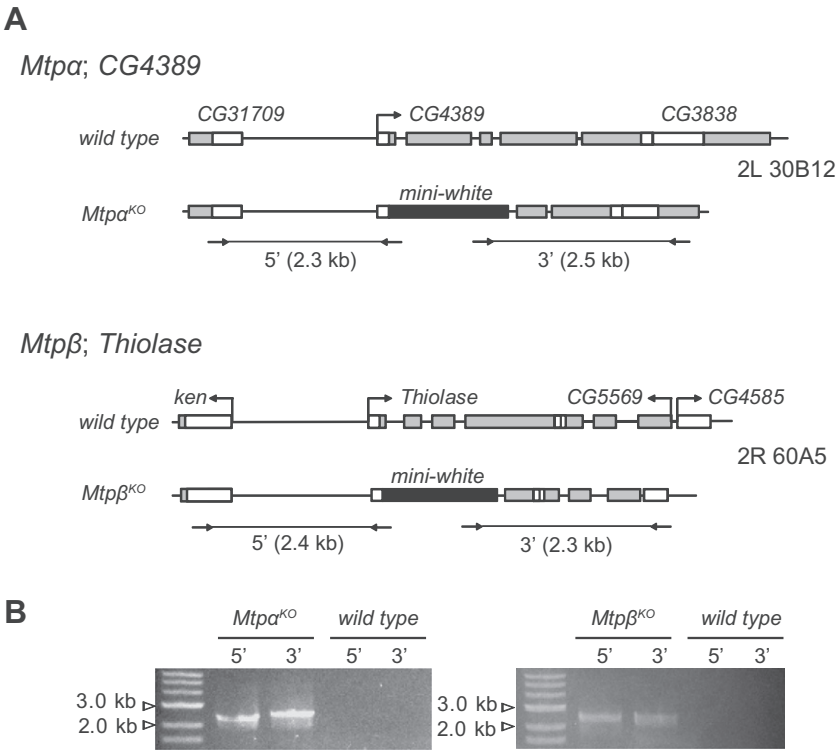

Fig. 1. Generation of *Mtpα*- and *Mtpβ*-knockout flies by gene targeting. (A) Genomic organization of the *Mtpα* and *Mtpβ* loci and the targeted mutant alleles. Gray and open boxes represent exons encoding the protein-coding and untranslated regions, respectively. Arrows with gene names indicate transcription start sites and the direction of transcription. Black lines bounded by arrows indicate the PCR assay fragments and primers, respectively. (B) Confirmation of correct targeting events by genomic PCR of the regions indicated in (A). Approximately 2.5-kb fragments could be amplified from genomic DNA of the mutant *Mtpα* and *Mtpβ* alleles, but not from that of the wild-type alleles.

2. Materials and methods

2.1. Fly strains and culture conditions

The y^1 w^{67c23} (*y w*) strain was used as a control. Flies were reared at 25 °C on standard glucose-yeast-agar media containing propionic acid and *n*-butyl *p*-hydroxybenzoate as mold inhibitors.

2.2. Generation of knockout flies

Mtpα- and *Mtpβ*-knockout flies were generated using the ends-out gene-targeting technique as described previously [17]. For constructing the targeting plasmids, approximately 2 kb of both the upstream and downstream regions of the *Mtpα* and *Mtpβ* loci were amplified by PCR with the following primers: *Mtpα*-5′ (forward: GCAATTAAATTCGGGGTCGAT, reverse: GAACTTTGATCATGCACCGTTAG); *Mtpα*-3′ (forward: ACTTCAAGAACGCAGACATCATC, reverse: AGCTAATTGACGACGTTGTAACC); *Mtpβ*-5′ (forward: GAAATATCCACCAACACACTGGT, reverse: CTTGCGAAAGTTTTCTGATTTCT); *Mtpβ*-3′ (forward: CTTCTGCTTGCATTATCATGACC, reverse: TGGCACTTAGACGTAGTCAACAA). The PCR-amplified fragments were subcloned into the polylinker of *p{EndOut2}* [17]. The mini–*white* gene was inserted between the upstream and downstream target sequences as a transformation marker. Targeted lines were backcrossed to the *y w* stock for six generations.

2.3. Lifespan measurement and starvation test

Twenty flies were kept in a glass vial containing standard glucose-yeast medium. Flies were transferred to fresh media every 1–3 days, and the number of dead flies was counted at the time of transfer. At least 90 flies were used for the lifespan assay for each genotype. For the starvation stress tests, 1-day-old flies were kept in vials containing 1% agar, and their survival was assessed every 8 h.

2.4. Climbing assay

Ten to twenty flies were placed in a graduated cylinder (2 cm in diameter; 20 cm in length), and gently bumped down to the bottom. Images were then captured at 8-s intervals, to measure the distance climbed up the cylinder by each individual. For each sample, ten trials were carried out to determine the average climbing activity.

2.5. Fecundity test

Three females and three males were placed in a vial containing standard medium for 22 h at 25 °C, and then transferred to a new vial every day. The number of eggs in each vial was counted. Six vials were used to determine the fecundity for each genotype.

2.6. Liquid chromatography-quadrupole time-of-flight mass spectrometry (LC–QTofMS)

Acylcarnitine and hydroxyacylcarnitine were quantified using a Waters (Milford, MA) LC–QTofMS system (LC: Acquity UPLC; MS: Xevo™ QTofMS). Ten males were collected and homogenized on ice in 100 µl of isopropanol/acetonitrile/10 mM NH_4Ac, pH 5.0 (1:1:1). The homogenates were centrifuged for 5 min at 2400×*g* and the supernatant was re-centrifuged for 5 min at 2400 × *g*. Fifty microliters of supernatant were recovered and transferred to a sample tube. The samples were applied to the LC–QTofMS system. Chromatographic separation was performed at 40 °C using an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm; Waters). The columns were equilibrated with H_2O containing 0.1% formic acid, and the compounds were eluted with an increasing gradient