A Cardiolipin-Binding Peptide Improves Cellular Bioenergetics in Mitochondrial Trifunctional Protein (TFP)-Deficient Mice and Patient Fibroblasts

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Abstract

Background: Current management of long-chain fatty acid oxidation disorders (LC-FAOD), including TFP deficiency (TFPD), is limited to nutritional management and triheptanoin. However, hospitalizations and significant morbidity remain a major problem for patients. The cardiolipin-binding peptide elamipretide has been shown to improve mitochondrial dysfunction in various conditions, including diabetes, heart disease, and mitochondrial respiratory chain (ETC) deficiencies, by stabilizing oxidized cardiolipins (CL) and thus the FAO/ETC macromolecular energy complex (MEC). TFP has recently been shown to have a fourth enzymatic activity, functioning in the maturation of CL. Of note, the clinical picture of TFPD is distinct from other LC-FAODs with peripheral neuropathy and retinopathy, which overlap those seen in ETC Complex I deficiency. We propose that CLBPs will stabilize MECs and improve bioenergetics in TFPD mice and patient fibroblasts.

Methods: Mice with a point mutation in the TFP subunit gene *hadhb* were treated with an elamipretide-like CL-binding peptide (CLBP) or placebo, and their cold tolerance (a function of intact cellular bioenergetics) was determined. TFPD and long-chain 3-hydroxyacyl-CoA dehydrogenase-deficient (LCAHDD) patient fibroblasts were treated *in vitro* and cellular bioenergetics were measured, including whole cell FAO and FAO-ETC coupling. Western blotting of 2D BNGE/SDS-PAGE gels and BNGE in-gel activity stains were used to study the effect on the MEC.

Results: Treatment of TFPβD mice with CLBP led to improved cold tolerance and prevented hypoglycemia development. Impaired MEC in untreated mice were stabilized with CLBP as shown by 2D BNGE/SDS-PAGE gels. TFPD and LCHADD patient cells showed improved cellular bioenergetics with CLBP treatment.

Discussion: These findings identify CLBPs as potential therapeutic agents for TFP/LCHAD deficiency. Additional studies of CL metabolism in response to this agent will help clarify the mechanism of action of this compound.

Introduction

Eukaryotic cells efficiently generate energy through three mitochondrial metabolic pathways: fatty acid oxidation (FAO), oxidative phosphorylation (OXPHOS), and the tricarboxylic acid cycle (TCA). Of the three, FAO works by primarily breaking down fatty acids (FA). Therefore, disruption of FAO affects the body's overall energy production, as seen in FAO disorders (FAOD).

Long-chain FAODs (LC-FAOD) are caused by mutations in the genes for LC-FAO enzymes. This group of disorders affects nearly 3,500 people in the United States. Clinically, patients with LC-FAOD exhibit hypoglycemia, muscle pain, rhabdomyolysis, and cardiomyopathy, with serious complications leading to hospitalizations or early death. Patients with trifunctional protein-deficiency (TFPD) present with additional clinical presentations of peripheral neuropathy and retinopathy, which overlap patients with electron transfer chain (ETC) Complex I deficiency. Current therapy for LC-FAOD patients, and especially TFPD, are inadequate and morbidity and mortality remain an issue.

Recently, the CLBP elamipretide has been shown to improve mitochondrial dysfunction in various conditions, including diabetes, heart disease, and mitochondrial respiratory chain deficiencies by stabilizing oxidized cardiolipins (CL). This, in turn, stabilizes the inner mitochondrial membrane and the FAO/ETC macromolecular energy complex (MEC). More recently, TFP has been shown to exhibit a fourth enzymatic activity in the maturation of CL. Importantly, disruption of CL production seriously affects multiple body systems, as seen in patients with Barth syndrome (BTHS).

We hypothesize that CLBPs will stabilize the MECs and improve bioenergetics in LC-FAOD, especially TFPD. We evaluated this by assessing the effects of an elamipretide-like CLBP on mitochondrial structure and function in TFPβD mice and patient fibroblasts.

Methods

Mice Treatments and Cold Tolerance Test: Mice with a point mutation in the TFP subunit gene *hadhb* (TFPβD) were treated with an elamipretide-like CLBP (25, 50, 100 mg/kg/day IP) or placebo (PBS) for 7 days. Following treatment completion, mice were fasted overnight and underwent a cold tolerance test, which measures intact cellular bioenergetics through observing rescue from cold-induced hypothermia and hypoglycemia when exposed to 10°C for up to 4 hrs.

Cell Cultures and Treatment: TFPβD mouse hepatocytes were isolated and cultured at 37°C with 5% CO_2 for 5 hrs prior to CLBP treatment for 19 hrs. TFPD patient fibroblasts were cultured in complete DMEM at 37°C with 5% CO_2 and were incubated with CLBP (0, 120, 240, 480nM) for 24 hrs prior to assessment.

Whole Cell FAO Flux Analysis: Flux through the FAO pathway was quantified by production of ^3H_2O from [9,10-^{3}H(N)]-oleic acid conjugated to FA-free albumin in TFPβD mouse hepatocytes or TFPD patient fibroblasts.

FAO-ETC Bridging (Coupling) Activity Assay: To observe the effect of CLBP on the interactions of FAO and ETC, a bridging assay measuring flux through FAO-ETC was performed. The reduction of cytochrome *c* was measured spectrophotometrically in TFPβD mice mitochondrial extracts that were incubated with palmitoyl-CoA (C16-CoA).

Blue-Native Gel Electrophoresis (BNGE), SDS-PAGE, 2D Western Blot (WB): To study the effect of CLBP on the MEC, analysis by BNGE, SDS-PAGE, and 2D WB were performed on TFPβD mice mitochondrial extracts. Protein density was measured and calculated using Bio-Rad Image Lab.

Acylcarnitine Profile Analysis: Blood serum samples collected from CBLP-treated and PBS-treated mice following cold tolerance tests were processed by the UPitt's Dept of Pediatric Metabolic Core according to their protocol to determine acylcarnitine concentrations.

Results

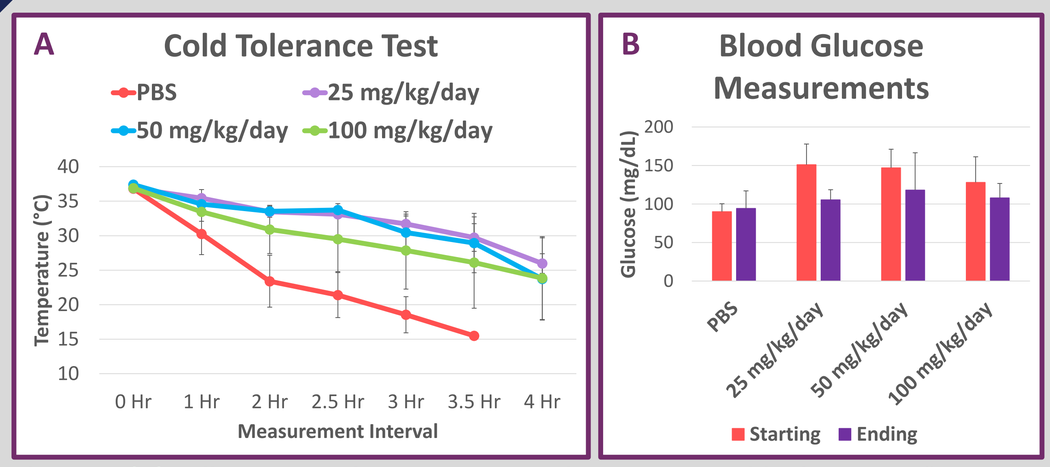


Figure 1. (A) Following a 7-day treatment with CLBP at variable doses, TFPβD mice were able to survive the 4 hr cold period while maintaining normal blood glucose levels **(B)**. Mice treated with placebo (PBS), however, were sacrificed early to prevent them from succumbing to the cold.

Figure 2. Isolated TFPβ mouse hepatocytes showed improved whole cell oxidation of oleate by nearly two-fold following treatment with CLBP at variable doses.

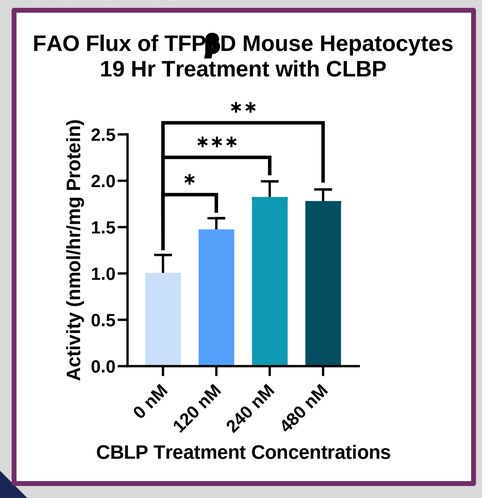


Figure 3. Treatment of TFPβD mice with CLBP restored bridging (coupling) activity from FAO to ETC by up to 20%, as shown in isolated heart mitochondria incubated with C16-CoA as substrate.

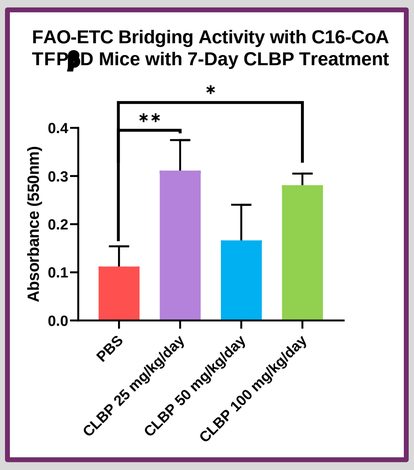
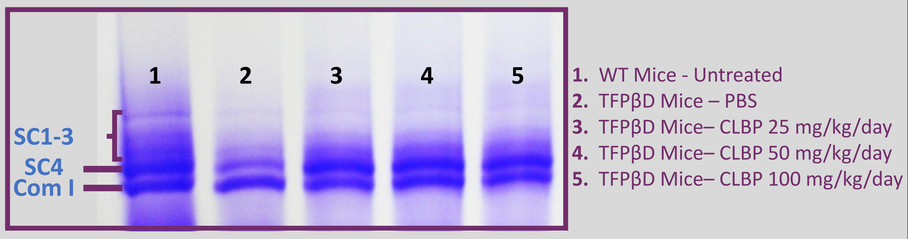


Figure 4. Coomassie blue staining of BNGE revealed PBS-treated TFPβD mice showed drastic decrease (~70%) in the amount of SC when compared to WT, while treatment with CLBP yielded up to 50% increased total SC protein density. Of note, SC4 was restored by ~60%.



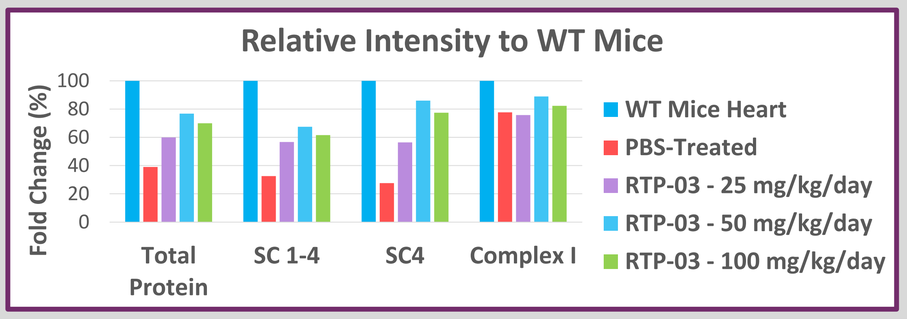


Figure 5. TFP typically migrates with the ETC-SC in mitochondrial extracts (A, D). TFPα/β is decreased in TFPβD mice heart mitochondrial extracts (B) but is restored by up to 25% following treatment with CLBP (C).

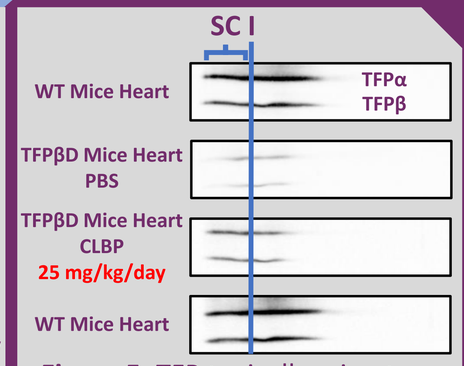


Figure 6. Acylcarnitine profiling of TFPβD mice blood serum revealed decrease (~50%) in hydroxylated long-chain (LC) acylcarnitines following treatment with CLBP.

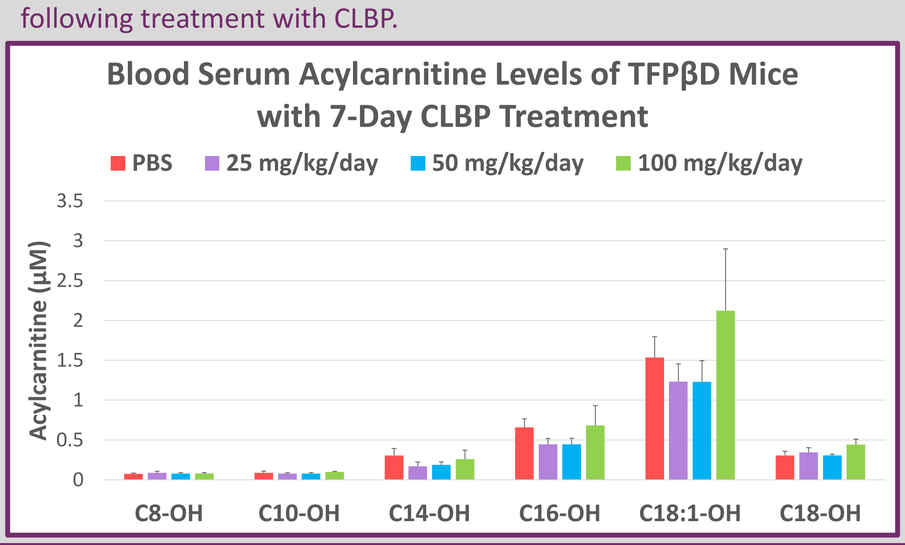
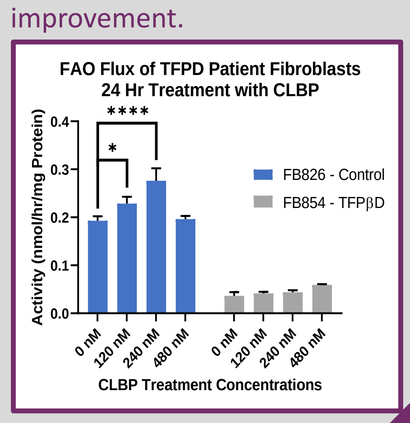


Figure 7. TFPβD patient fibroblasts (FB854) treated with CLBP at variable doses for 24 hrs showed minimal improvement in whole cell oxidation of oleate, although control cells demonstrated significant improvement.



Discussion

Current management options for TFP deficiency (TFPD) do not address the underlying pathophysiology of the disorder. Through functional assays, we have demonstrated that the elamipretide-like CL-binding peptide (CLBP) improves bioenergetics in TFPβD mice. However, the effect of the CLBP is variable in TFPD patient fibroblasts likely due to allelic heterogeneity in this disorder. Assays of TFPD mice show that CLBP improves assembly of ETC-SC and restores the normal interaction of TFP with the ETC-SC, allowing for restored channeling of reducing equivalents from FAO to the ETC. Our findings identify CLBP as a treatment option for TFPD and suggest that it may be be effective for other LC-FAODs.

References

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