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Mitochondrial Trifunctional Protein Defects: Clinical Implications and Therapeutic Approaches

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Abstract

The mitochondrial trifunctional protein (MTP) is a heterotrimeric protein that consists of four α -subunits and four β -subunits and catalyzes three of the four chain-shortening reactions in the mitochondrial β -oxidation of long-chain fatty acids. Families with recessively inherited MTP defects display a spectrum of maternal and fetal phenotypes. Current management of patients with MTP defects include long-term dietary therapy of fasting avoidance, low fat/high carbohydrate diet with restriction of long chain fatty acid intake and substitution with medium chain fatty acids. These dietary approaches appear promising in the short-term, but the long-term outcome of patients treated with dietary intervention is largely unknown. Potential therapeutic approaches targeted at correcting the metabolic defect will be discussed. We will discuss the potential use of protein transduction domains that cross the mitochondrial membranes for the treatment of mitochondrial disorders. In addition, we discuss the phenotypes of MTP in a heterozygous state and potential ways to intervene to increase hepatic fatty acid oxidative capacity.

Keywords

fatty acids; β -oxidation; mitochondrial trifunctional protein; LCHAD; mitochondria; TAT; PTD; fusion proteins; protein transduction domain; cell penetrant peptide

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1. INTRODUCTION

Mitochondrial β -oxidation of fatty acids consists of multiple transport steps and four enzymatic reactions resulting in the sequential removal of two-carbon, acetyl-coenzyme A units. The mitochondrial trifunctional protein (MTP) is a heterotrimeric protein that consists of four α -subunits and four β -subunits and catalyzes three of the four chain-shortening reactions in the mitochondrial β -oxidation of long-chain fatty acids. Defects in mitochondrial function cause serious pediatric and maternal morbidity and mortality. Mitochondrial fatty acid oxidation (FAO) disorders are recessively inherited, and defects in FAO are estimated to affect ~1 in 10,000 [1]. The estimated prevalence of MTP defects in association with fetal MTP defects in the US is 1 in 38,000 pregnancies [2]. We have generated a mouse model for a null mutation causing complete MTP deficiency [3], with fetuses that accumulate long chain fatty acid metabolites identical to the human deficiency. These homozygous MTP defect mice suffer neonatal hypoglycemia and sudden death 6–36 hours after birth. We also have recently documented that aging mice heterozygous for the MTP defect (MTPa^{+/-}) causes development of insulin resistant and hepatic steatosis [4]. Current clinical therapy for mitochondrial disease focuses more on the treatment of symptoms instead of correction of the actual defective mechanism. In this review we discuss the role of mitochondrial β -oxidation, consequences of defective mitochondrial β -oxidation, current therapies and novel therapeutic approaches for the treatment of mitochondrial defects.

2. MITOCHONDRIAL FATTY ACID β -OXIDATION

Fatty acid oxidation (FAO) is the major source of energy for skeletal muscle and the heart, while the liver oxidizes fatty acids primarily under the conditions of prolonged fasting, during illness, and during periods of increased physical activity. FAO also plays an essential role in the intermediary metabolism of the liver. Hepatic FAO fuels gluconeogenesis and the synthesis of ketone bodies, 3-hydroxy butyrate and acetoacetate, which are utilized as alternative sources of energy by extrahepatic organs, like the brain when blood glucose levels are low [5].

Mitochondrial β -oxidation of fatty acids consists of multiple transport steps and four enzymatic reactions resulting in the sequential removal of two-carbon, acetyl-coenzyme A units. Plasma long chain fatty acids are transported actively across the plasma membrane, esterified to coenzyme A, carried by fatty acid binding proteins through the cytoplasm to the mitochondria, and translocated across the mitochondrial inner membrane by the carnitine shuttle to the mitochondrial matrix. Once in the mitochondrial matrix, the fatty acid is sequentially cleaved, two carbons shorter, by the four reactions of the β -oxidation spiral. Each step in the spiral is catalyzed by 2-4 distinct enzymes, encoded by separate nuclear genes that exhibit different overlapping substrate specificities. The first step in the spiral, as shown in Figure 1, is an acyl-CoA dehydrogenase reaction, catalyzed by very long-chain acyl-CoA dehydrogenase (VLCAD) and its homologous enzymes, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD) or short-chain acyl-CoA dehydrogenase (SCAD). The second step in the pathway adds a water across the double bond and is catalyzed by either a long-chain 2,3-enoyl-CoA hydratase (LCEH) or a short-chain 2,3-enoyl-CoA hydratase (SCEH) which hydrates the 2,3 enoyl-CoA across the double bond. The third step is catalyzed by a long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) or a short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) which oxidizes the 3-hydroxy position producing a 3-ketoacyl-CoA. The fourth and last step in the spiral is mediated by a long-chain 3-ketoacyl-CoA thiolase (LKAT), medium-chain 3-ketoacyl-CoA thiolase (MKAT), or short-chain 3-ketoacyl-CoA thiolase (SKAT), that shortens the fatty acyl-CoA substrate by two carbons by cleaving off acetyl-CoA. For long chain length fatty acids, the last three steps are mediated by the enzyme complex mitochondrial trifunctional protein (MTP) [6,7]. The shortened acyl-CoA can then reenter the fatty acid β -oxidation spiral until the fatty acid is completely broken down

into a 2-carbon or 3-carbon species. The acetyl-CoA produced can be used for ketogenesis, steroid genesis and as a substrate for the tricarboxylic acid cycle.

3. DISORDERS IN MITOCHONDRIAL β -OXIDATION

FAO disorders have become an important group of inherited metabolic disorders characterized by a wide array of clinical presentations and important causes of pediatric and maternal morbidity and mortality. More than 20 different disorders that affect β -oxidation have been identified. LCHAD deficiency was first described in 1989 [8] and MTP deficiency was first reported in 1992 [9]. In 1992, two groups of investigators, independent of each other, reported that LCHAD was part of the MTP enzyme complex, which is associated with the inner mitochondrial membrane [10,11]. In isolated LCHAD deficiency, the pathway is blocked after the enoyl Co-A hydratase reaction and before the 3-hydroxyacyl Co-A dehydrogenase reaction, causing the accumulation of medium- and long-chain 3-hydroxy fatty acids and their metabolites. In complete MTP deficiency, the pathway is blocked after the acyl Co-A dehydrogenase reaction and before the enoyl Co-A dehydrogenase reaction causing the accumulation of straight chain fatty acids and their metabolites.

Defects in mitochondrial function cause serious pediatric morbidity and mortality. Mitochondrial FAO disorders are recessively inherited, and defects in FAO are estimated to affect ~1 in 10,000 [1]. Postmortem biochemical studies suggest that 1-5% of sudden unexpected infant deaths are attributed to undiagnosed FAO disorders [12-14]. Children affected by these recessively inherited disorders usually present within the first year of life with nonketotic hypoglycemia, hepatic dysfunction, and/or skeletal and cardiac myopathy [15], which may progress to coma and death if untreated [16]. Our group has reported on the α -subunit molecular defects and phenotypes in 35 patients with documented isolated LCHAD deficiency or complete MTP deficiency [2,17,18]. Patients with complete MTP deficiency present predominantly with hepatic steatosis, cardiomyopathy, skeletal myopathy and neuropathy, while patients with LCHAD deficiency presented predominately with a serious liver phenotype. We and others have documented that women who carry fetuses with MTP defects often develop acute fatty liver of pregnancy (AFLP) and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome [2,18,19]. AFLP has an incidence of 1 in 13,000 deliveries [20] and affects women of all ages and race. The cause of AFLP is unknown and recent molecular advances suggest that AFLP may result from mitochondrial dysfunction. Maternal liver disease occurs in ~60% of women who carry fetuses with MTP α -subunit defects [2] and in about 15% of women who carry fetuses with MTP β -subunit defects [21]. The precise mechanism by which fetal deficiencies in β -oxidation influence maternal status is not fully understood and warrant future investigation.

4. MITOCHONDRIAL TRIFUNCTIONAL PROTEIN DEFECTS

MTP is a heterotrimeric protein that consists of four α -subunits and four β -subunits and catalyzes the last three steps in mitochondrial long-chain fatty acid β -oxidation. The α -subunit amino-terminal domain contains the long-chain 3-enoyl-CoA hydratase enzymatic activity while the LCHAD enzymatic activity resides in the carboxy-terminal domain. The β -subunit has the long-chain 3-ketoacyl-CoA thiolase enzymatic activity. The association of the α - and β -subunits to form the enzyme complex is necessary for membrane translocation and for the catalytic stability of the 3 enzymes [22,23].

MTP defects have recently emerged as an important group of errors of metabolism because of their clinical implications. Human defects in the MTP complex are recessively inherited and cause either isolated LCHAD deficiency, with normal or partially reduced thiolase and hydratase activity, or complete MTP deficiency with markedly reduced activity of all 3 enzymes. Patients have been described with either an isolated LCHAD deficiency or MTP

deficiency. Based on our previous reports, the majority of patients have been described as having isolated LCHAD deficiency [2]. Since being first described in 1989 [8], isolated LCHAD deficiency is recognized as one of the more severe FAO disorders. The estimated prevalence of MTP defects in association with fetal MTP defects in the US is 1 in 38,000 pregnancies [2].

Our group and others have attempted to characterize normal and mutated MTP genes utilizing molecular approaches. Two teams, independent of each other, delineated the G1528C mutation in exon 15 of the MTP α -subunit which alters amino acid 474 from glutamic acid to glutamine (E474Q) and replaces the acidic and negatively charged side-chain with a neutral, amide containing residue [24,25]. The likely mechanism by which the E474Q mutation causes isolated LCHAD deficiency is that the mutation inactivates LCHAD directly within the catalytic domain, and hence, preserves the other MTP enzyme activities. The likely mechanism has been elucidated by Barycki and colleagues [26] based on the crystal and structural analysis of x-ray diffraction data from the human SCHAD which is highly homologous to the LCHAD domain. E170 in SCHAD, a residue that is analogous to LCHAD E474, is located in the NAD-binding domain within the active catalytic site. E170 is also in a position to interact with another residue, H158 which serves as a base abstracting a proton from the 3-hydroxy group of the substrate. Substitution of E170 in SCHAD with glutamine disrupts the electrostatic interaction between E170 and H158, which is essential for catalysis. Similar to what happens to E170Q mutation in SCHAD, the E474Q mutation causes isolated LCHAD deficiency and blocks the β -oxidation resulting in the accumulation of 3-hydroxy fatty acid metabolites.

5. MTP DEFICIENT MOUSE MODEL

We have generated a mouse model for a null mutation causing complete MTP deficiency [3]. Gene targeting was used to generate an MTP α -subunit null allele and produce mice that fetuses accumulate long chain fatty acid metabolites identical to the human deficiency. The homozygous (*MTP α ^{-/-}*) mice lack both MTP α - and β -subunits, have biochemical changes identical to those of human deficiency, have low birth weight compared to +/- and +/+ littermates, and suffer neonatal hypoglycemia and sudden death 6–36 hours after birth. Analysis of the histopathologic changes in the *MTP α ^{-/-}* pups revealed rapid development of hepatic steatosis after birth, followed by acute degeneration and necrosis of the cardiac and diaphragmatic myocytes, which may be the reason for the underlying etiology for sudden death [3]. We document that intact mitochondrial long chain FAO is essential for fetal development and for survival after birth as expression studies of hepatic MTP revealed a significant increase in MTP expression that reach adult MTP levels within days after birth. This suggests a reliance on FAO as a source of energy. This also coincides with the neonatal metabolic switch in nutritional fuel from glucose to maternal breast milk which has a high fat content. The lack of maternal phenotype in the MTP deficient mouse model is consistent with our earlier observation in families with MTP mutations.

6. PHENOTYPES ASSOCIATED WITH MTP HETEROZYGOSITY

Our lab group has recently documented that aging mice heterozygous for the MTP defect (*MTP α ^{+/-}*) causes development of insulin resistance and hepatic steatosis [4]. We documented an age-dependent decline in hepatic MTP expression in our mice [4], and provided, for the first time, direct evidence that genetic impairment of mitochondrial β -oxidation of fatty acids causes insulin resistance in mice. *MTP α ^{+/-}* mice developed insulin resistance, as evidenced by increases in insulin and glucose area under curve following glucose and insulin tolerance tests, and hepatic steatosis at 9–10 months of age when fed a normal rodent chow diet. Figure 2 shows a representative liver section from an *MTP α ^{+/-}* mouse compared with a wild-type littermate control. This figure demonstrates the abundance of microvesicular and macrovesicular lipid

droplets stained with Oil-Red O in association with defective MTP [4]. These genetically engineered mice with impaired FAO secondary to a defect in MTP also displayed hepatic mitochondrial abnormalities. Figure 3 shows an example of the ultrastructural changes in the mitochondrial in an $MTPa^{+/-}$ mouse compared with a wild-type littermate control. MTP heterozygous animals also had higher antioxidative activity of superoxide dismutase and glutathione peroxidase, and increased expression of cytochrome P-450 2E1, findings consistent with increased hepatic oxidative stress. We also have preliminary data to suggest that a high-fat diet in combination with the genetic defect induces hepatic steatosis at a much earlier age in the $MTPa^{+/-}$ mice and further increases reactive oxygen species formation in these animals (J.A. Ibdah, unpublished results). Our findings suggest that combination of heterozygosity for the MTP defect and an age-associated decline in MTP expression causes development of hepatic steatosis and insulin resistance in our aging $MTPa^{+/-}$ mice.

7. CURRENT MANAGEMENT OF MTP DEFECTS

It is without question that FAO disorders associated with mitochondrial dysfunction affect a significant portion of the population. However, current clinical therapy for mitochondrial disease focuses more on the treatment of symptoms instead of correction of the actual defective mechanism. The current management of MTP defects relies almost entirely on dietary interventions. The main stay of long term dietary therapy in disorders of FAO is fasting avoidance. Generally, a low-fat, high-carbohydrate diet with approximately 70% of calories from carbohydrate, 15% from protein, and 15-20% from fat is instituted, with restriction of long chain fatty acid intake. Another dietary therapy is the substitution of long chain fatty acids with medium chain fatty acids [27,28]. In addition, the supplementation of L-carnitine has been shown to be effective in disorders with primary carnitine deficiency, as it corrects the defect in hepatic ketogenesis and improves cardiac function and muscle strength [29]. Findings from its use in the treatment of FAO disorders are mixed. Carnitine supplementation has the potential benefit of detoxifying accumulated acyl-CoA intermediates and replenishing the intramitochondrial carnitine pool; however, carnitine supplementation has been questioned in long-chain FAO defects because of the accumulation of long-chain fatty acylcarnitines that may cause cardiac arrhythmias [30].

These nutritional strategies improve survival and prognosis in patients with MTP defects. Adherence to strict dietary protocol over one year reduced metabolic decompensation and prevented hospitalization in 10 children with LCHAD or MTP deficiency [31]. While the short-term effects of dietary interventions appear promising, the long-term prognosis of patients with β -oxidation defects remains unknown. Many LCHAD and MTP deficient patients develop retinal problems and peripheral neuropathy. Rhabdomyolysis (breakdown and loss of skeletal muscle) is a frequent complication of LCHAD and MTP deficiency. The accumulation of acylcarnitines and hydroxyacyl fatty acid metabolites are thought to have toxic effects on skeletal muscle and contribute to this condition. A recent study by Gillingham et al [32] provide some evidence for the beneficial combination of medium chain fatty acid supplementation and exercise in improving metabolic control in children with LCHAD and MTP deficiencies. Bypassing the MTP defect with medium chain fatty acid supplementation provides essential energy for the patient, and the medium chain fatty acids are preferentially oxidized by skeletal muscle, and the accumulation of cytotoxic long-chain fatty acid metabolites is inhibited.

8. POTENTIAL THERAPIES FOR MTP DEFECTS

Due to the plethora of identified mitochondrial diseases and their associations with virtually all metabolic disorders, the mitochondria represent a promising target for drug therapy. However, delivery of drugs and therapeutic compounds is primarily limited by their ability to penetrate the cell membrane. The bioavailability of compounds targeted to intracellular sites

depends on the conflicting requirements of being sufficiently polar for administration and distribution, yet non-polar enough to diffuse through the non-polar lipid bilayer of the cell [91]. In addition, the molecular weight of most drugs that can easily traverse the lipid membrane is approximately 500 Da [33]. Thus, most successful compounds have narrow physical characteristics. Many promising drugs fail because they fall outside this range and efforts to make them available may be toxic. In addition to this, many sites of action for presumed therapeutic compounds, such as enzymes or regulatory proteins, require processing and targeting of the compound once inside the cell. For example, regulation of DNA transcription requires nuclear localization of the therapeutic compound, and repair of a mitochondrial defect requires targeting and localization within the correct compartment of the mitochondria. This step alone represents a significant hurdle to the development of many strategies to repair defects within a cell. Yet many metabolic diseases are potential candidates for gene therapy because in many cases the disease is caused by a mutation in a single gene and has been clearly identified. Approximately 15% of all registered gene transfer trials in the United States have been designed for the treatment of metabolic and genetic diseases [34].

Two distinct genetic systems encode mitochondrial proteins: mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Of the hundreds of proteins that are found in the mitochondria, the mitochondrial genome encodes only 13 of these and the rest must be imported from the cytosol [35,36]. Defects in nuclear encoded proteins or protein import into the mitochondria result in human disease [37]. Numerous investigators have attempted to develop methods sufficient to target drug delivery to the mitochondria. There are two broad categories of gene therapy: viral and non-viral.

8.1 Viral Gene Therapies

There are three common types of viral vectors being explored for their therapeutic effects: retroviral, adenoviral, and lentiviral vectors. Viral vectors are an attractive therapy because they are efficient in transducing most cells and have been used extensively as tools for studying basic biological phenomena. The retroviral vector system is comprised of two components: the transfer vector which carries the cis-acting elements necessary for replication and integration of the viral DNA and the packing cell line containing all of the structural and catalytic functions of the virus. The goal of gene therapy for genetic diseases is to bring about long-lasting expression of the missing or defective gene. Retroviruses represent a long-term treatment because they insert themselves into the host chromosomes and become part of the cell's regularly replicated DNA. However, retroviral genes are inserted in the genome randomly, which carries the risk of insertional mutagenesis [38]. Retroviral vectors have been used to correct liver disease in a wide variety of species *ex vivo* [39]. However, because hepatocytes do not actively proliferate under physiological conditions, there is low efficiency of gene transfer *in vivo* due to difficulty in transduction.

An alternative vector commonly used in research is adenoviral vectors. One of the primary benefits of adenoviral vectors over retroviral is that adenoviral vectors efficiently transduce into non-dividing cells, such as hepatocytes. Multiple studies have demonstrated the successful use of recombinant adenoviral vectors to achieve *in vivo* gene expression [34]. However, because these vectors do not integrate into the genomic DNA, the host's immune response against the viral protein causes clearance of the DNA and the overall expression is quite transient. This requires repeated treatments and subsequently increases the immune response. One potential foreseen use for adenoviral vectors may be to induce transient high-level gene expression during an acute metabolic crisis [34]. In addition, newly developed versions of adenoviral vectors appear to provide longer transgene expression [34].

A third alternative vector is the lentiviral vectors. Lentiviruses are a class of retroviruses with unique characteristics. Lentiviral vectors, like adenoviruses, are able to transduce into non-

dividing cells, such as hepatocytes. Human immunodeficiency virus (HIV) is the best known and studied virus of this family, and HIV-1–derived lentiviral vector systems have been developed. Envelope glycoproteins of lentiviruses bind to cell surface receptors and allow entry into the cytoplasm. The envelope glycoproteins also can be adjusted so that the tropism to certain target populations increases [40]. Once in the cytoplasm, the viral RNA is reverse transcribed to cDNA, called a provirus. The provirus translocates into the nucleus, integrates into the host chromosome and then is permanently expressed. One advantage of lentiviruses is that they can deliver 8 kb or more DNA to target cells. In addition, recent modifications have improved their biosafety, making them a candidate for treating hepatic diseases [39]. Lentiviruses also seem to be a potential candidate vector system for stable transduction of fetal stem cells.

8.2 Non-Viral Gene Therapies

Non-viral therapeutic techniques are appealing due to their simplicity. Non-viral agents usually do not require integration or promotion, and non-viral vectors may be more suitable, in part, due to the lack of a specific immune response. Most mitochondrial disorders are caused by nuclear gene defects. The majority of the nuclear encoded proteins are targeted to the mitochondria by the use of presequences at the N-terminus of the precursor protein [41]. The entry of these mitochondrial targeting sequences (MTS) into the mitochondria is receptor mediated and utilizes the outer (TOM) and inner (TIM) membrane translocation machinery for passage through the mitochondrial membranes [42,43]. Upon recognition of the MTS, chaperon proteins temporarily unfold the protein. Once in the mitochondria, the MTS (typically 10-70 amino acids) is removed by 1 or 2 proteolytic steps and the transit peptide is cleaved by the mitochondrial processing peptidase. This processing yields the protein in the refolded, mature, and active form.

The use of MTS-fused proteins for mitochondrial gene therapy has been shown via transfection into cultured cells. These studies have shown that this method allows not only targeting of the fusion protein to mitochondria, but it is also processed, allowing for complete localization and functionality of the fused protein [44,45]. However, due to the same limitations incurred by other gene therapies, in vivo use of this technology has been limited. Delivery of MTS-fused proteins may be perturbed in conditions where defects in mitochondrial protein import exist [46] and often proteins encoded in mitochondrial DNA are too hydrophobic for the required maintenance of protein unfolding during transit [47].

8.3 TAT-MTS Fusion Proteins

It has long been known that positively charged, or cationic peptides, will cross cell membranes independent of receptors or specific transport mechanisms. Recently, a novel strategy for delivery of synthetic compounds has been described and is being actively investigated by both industry and academic researchers [48]. To overcome the limitations of viral vectors for delivering gene products to mitochondria, our group used protein transduction domains (PTD), also known as cell penetrant peptides, which cross cell membranes to deliver fusion proteins to mitochondria. A major benefit of the use of PTDs is that they can rapidly translocate into the cytosol and can deliver macromolecules to mitochondria through pathways independent of the MTS and mitochondrial import pathways. Our group has previously summarized findings from multiple investigators demonstrating that attachment of nucleic acids, peptides, and even large proteins, to PTDs allows for efficient transduction across all cell membranes [49].

One of the PTD derived from the human immunodeficiency virus-1, TAT peptide, consists of 11 amino acids including 6 arginine and 2 lysine residues. TAT (transactivator of transcription) peptides have the ability to move (transduce) attached peptides, large proteins, and nucleic acids across virtually all cell membranes in a non-receptor mediated fashion allowing delivery

of cargos, such as drugs and biologically active proteins, to mitochondria in tissues and cells in culture. TAT peptide is well tolerated [50] with only minimal detrimental effects seen at high concentrations in cell culture [51,52]. Indeed, TAT has recently been used to deliver a cytosolic protein to rescue the defect of the purine nucleoside phosphorylase deficient mouse [53]. This was the first report that TAT could be used to rescue a genetic defect in an animal model. However, because TAT fusion proteins follow a concentration gradient, their use as therapeutic vehicles may be limited by the loss of TAT fusion protein from the cell if it is not bound or processed.

One strategy to improve delivery and retention of a TAT-mediated fusion protein is to separate the TAT from the protein of interest by placing a cleavable sequence in between the two. Our research group has combined the technology of the TAT-PTD with the use of MTS fusion peptides to efficiently deliver proteins to cells and localize them within mitochondria. The MTS serves as a place within the fusion protein that allows for separation of the protein of interest from TAT by the mitochondrial matrix processing peptidase. Del Gaizo and co-workers constructed a TAT fusion protein consisting of the MTS derived from mitochondrial malate dehydrogenase (mMDH) and the green fluorescent protein (TAT-mMDH-GFP). They have shown that this complex is able to not only cross cell and mitochondrial membranes, but that when the protein reached the mitochondrial matrix, the mMDH-MTS is recognized and cleaved [51,52]. Once cleavage occurs, the TAT peptide is separated from GFP, effectively trapping GFP in the matrix. Rapoport and colleagues have followed up on these findings to show that TAT can deliver lipoamide dehydrogenase (LAD) into mitochondria to correct the metabolic defect in fibroblasts from patients with LAD deficiency [54].

Further experiments have shown that TAT can be used with other MTS to deliver proteins to other compartments within mitochondria. In Figure 4, the TAT peptide was genetically fused to the MTS for sarcomeric mitochondrial creatine kinase. This MTS targets the creatine kinase to the intermembranous space of the mitochondria. This construct was then genetically fused with a fluorescent protein, DsRed.T3, to form TAT-sMtCK-DsRed.T3. When the purified fusion protein was applied to cells in culture, the TAT-sMtCK-DsRed.T3 localized within the mitochondria as shown by the co-localization with the mitochondria-specific dye, CMX-Green (Molecular Probes). Since DsRed.T3 forms a tetramer to generate fluorescence, it is clear that TAT fusion proteins can deliver complex, multi-subunit proteins that must re-fold and interact with other proteins.

8.4 MTP Defects and TAT-MTS Fusion Proteins

To test the hypothesis that TAT-fusion proteins could be used to deliver proteins to a fetus, our group injected pregnant mice at 18-19 days of gestation were injected intraperitoneally with 2 mg/kg of TAT-mMDH-GFP. Our analysis revealed strong GFP signal in maternal and fetal livers 24 hours post injection. Pups from these experiments continued to show strong GFP fluorescence in liver 7 days after maternal injection (reviewed in [49]). Analysis from other mitochondrial rich tissues from these animals, such as brain and heart, revealed similar localization of the signal sequence [51,52].

These findings indicate that TAT-fusion proteins can cross the placental barrier and are detectable in the fetus and newborn pups. These findings also offer the possibility of treating or preventing the complications caused during pregnancy of a mother carrying an affected fetus by the administration of a TAT-MTP fusion protein to replace the defective protein and restore function. Both the α - and β -subunits of MTP are nuclear encoded mitochondrial proteins and therefore contain their own MTS, making a fusion protein with TAT relatively straightforward to construct. This represents an exciting prospect; however, MTP is a multifaceted protein, unlike the exogenous protein GFP. It is quite possible that the TAT-MTP protein may not fold

properly or dimerize with the other subunit and remain inactive. If this is the case, alternative mitochondrial delivery strategies may need to be employed.

9. INCREASING MITOCHONDRIAL FATTY ACID β -OXIDATIVE CAPACITY

While complete MTP defects occurs in 1:38,000, it is estimated that 2%–3% of the US population is heterozygous for a defect in mitochondrial fatty acid oxidation [1]. This represents a substantial health risk for 8-12 million Americans, as heterozygosity for mitochondrial fatty acid defects cause inefficient mitochondrial β -oxidation and a progressive accumulation of intra-hepatic fatty acids. These figures likely contribute to the fact that ~34% of US adults have non-alcoholic fatty liver disease [55]. A challenge for health care professionals is to find means by which to increase the function in the defective mitochondria, increase mitochondrial biogenesis and overall mitochondrial content, or activate extra-mitochondrial pathways to circumvent the genetic defects in mitochondria under these conditions. We know of no evidence to date to support the use of therapeutic interventions for increasing hepatic MTP expression or the stability of the nuclear encoded genes. However, potential therapeutic targets for increasing mitochondrial fatty acid oxidative capacity of the liver are the peroxisome proliferator-activated receptor- γ coactivator 1 (PGC-1) family members and the nuclear hormone receptor family of transcription factors, peroxisome proliferator-activated receptors PPAR α and PPAR γ .

Hepatic fatty acid oxidation occurs in three subcellular organelles, β oxidation in mitochondria and peroxisomes, and ω -oxidation in the endoplasmic reticulum (ER) [56,57]. Mitochondrial β -oxidation is the dominant oxidative pathway for the oxidation of short through long-chain fatty acids under normal physiologic conditions. Short-chain and medium-chain FFAs freely enter the mitochondria, while long-chain FFAs entry into the mitochondria is regulated by the activity of the enzyme carnitine palmitoyl transferase-1 (CPT-1). Peroxisomes are involved in the β -oxidation chain shortening of long-chain and very-long-chain fatty acyl-CoAs [58], and medium chain fatty acids (mainly lauric acid) activate CYP450A1 and stimulates ω -oxidation of fatty acids in ER [59]. Mitochondrial activity is transcriptionally controlled, in part, by the nuclear receptors and the PGC-1 related family. PGC-1 α potently induces the expression of genes implicated in energy homeostasis in almost every cell type through known mitochondrial regulators such as the estrogen-related receptors (ERRs), PPARs, or nuclear respiratory factors (NRF-1, 2). NRFs are transcription factors that stimulate several of the mitochondrial nuclear-encoded genes, including genes involved in oxidative phosphorylation and mitochondrial transcription factor A (Tfam), a key transcription factor for the mitochondrial genome. PGC-1 α and PGC-1 β are preferentially expressed in tissues with high oxidative capacity such as heart, skeletal muscle and brown adipose tissue, where they serve critical roles in the regulation of mitochondrial functional capacity and cellular energy metabolism. Liver PGC-1 α and PGC-1 β expression is low in normal conditions, but is upregulated with fasting and in response to metabolic stress [60-62].

Mitochondrial oxidative capacity is decreased in liver tissue of patients and animal models with hepatic steatosis [63,64], and hepatocytes from PGC-1 α deficient mice have diminished fatty acid oxidative capacity and mitochondrial respiration rates [65]. In addition, both prediabetic and diabetic subjects exhibit significant reductions in PGC-1 α expression [66]. In contrast, peroxisomal β oxidation is elevated and activated in diabetic liver and liver from fatty rats [67-70]. Since peroxisomal fatty acid entry is not carnitine dependent or sensitive to inhibitors of CPT-1, it is thought that mitochondria and peroxisomes work in concert to prevent lipid accumulation in a high-fat environment [67,68].

Nuclear receptors PPAR α and PPAR γ are pleiotropic regulators of glycolytic and oxidative metabolism, and are major candidates for the enhancement of hepatic fatty acid oxidation.

Hepatic PPAR α targets acyl-CoA oxidase [71,72], the rate limiting step in peroxisomal β -oxidation, and is thought to promote fatty acid oxidation mainly through changes in the expression of genes involved in peroxisomal fatty acid oxidation and increased peroxisomal proliferation in liver [73,74]. Targeting peroxisomal fatty acid oxidation is an exciting prospect in the prevention of fatty liver disease and could potentially circumvent mitochondrial defects. However, this system may be costly as the rate limiting step in peroxisomal β -oxidation, fatty acyl-CoA oxidase, generates hydrogen peroxide resulting in reactive oxygen species (ROS) that may overwhelm cellular defense systems [59]. It is thought that ROS generation activates inhibitor κ B kinase (IKK) and JNK-1 pathways, which may limit activation of the Akt pathway and lead to other metabolic disorders, including hepatic insulin resistance [75]. It is likely that anti-oxidative enzyme systems, such as glutathione peroxidase and superoxide dismutase, would need to be upregulated in concert with peroxisomal fatty acid oxidation in order for beneficial effects to be realized.

The most widely utilized pharmacological interventions for the treatment of type 2 diabetes represent attractive tools for the modulation of hepatic mitochondrial and extra-mitochondrial fatty acid oxidation; these include the PPAR agonists, fibrates and thiazolidinediones (TZDs), and the AMP kinase (AMPK) activator, metformin. It has been shown that PPAR agonists (fibrates-PPAR α ; TZDs-PPAR γ) not only improve insulin sensitivity by suppressing the ectopic storage of lipids into liver, but these agents also increase genes involved in hepatic fatty acid oxidation [76]. In addition, while the precise mechanism(s) of action are not completely known, metformin targets and activates AMPK [77], which has multiple beneficial effects. Administration of metformin reduces hepatic gluconeogenesis, decreases absorption of glucose from the gastrointestinal tract, and increases insulin sensitivity. Metformin also has been shown to reduce hepatic lipogenesis and increase fatty acid oxidation in the *ob/ob* mouse model of hepatic steatosis [78]. Furthermore, metformin use in non-diabetic patients with hepatic steatosis reduces liver fat by 50% and decreases liver inflammation and necrosis [79], suggesting a role for enhanced fatty acid oxidation. Additional studies need to be performed to examine the effects of PPAR agonists and metformin on separating the effects on mitochondrial and extra-mitochondrial pathways.

Exercise represents another means to potentially increase mitochondrial function and relieve some of the hepatic stress associated with primary mitochondrial defects. Exercise increases fatty acid oxidation by increasing mitochondrial biogenesis in skeletal muscle [80] through PGC-1 α 's coactivation of NRF-1 and NRF-2, and increased expression of Tfam and by improving the oxidative capacity of existing mitochondria through increased expression of CPT-1. The positive effects of PGC-1 α upon mitochondrial fatty acid oxidation, as well as its capacity to increase the activity and protein contents of molecules involved in the TCA cycle and oxidative phosphorylation, demonstrates its robust capacity for increasing cellular respiration. These findings may be similar in liver, but remain unexplored.

AMPK is known to be activated by exercise and it has been shown that AMPK can activate mitochondrial biogenesis through MEF2- and CREB-mediated increased PGC-1 α expression [81]. In the liver, activation of AMPK results in decreased production of plasma glucose, cholesterol, triglyceride and enhanced fatty acid oxidation. The two leading diabetic drugs, namely metformin and rosiglitazone show their metabolic effects partially through AMPK. Previous studies have demonstrated the ability of exercise to increase hepatic AMPK activity levels in rodents [82,83]; whereas, we have previously demonstrated that exercise increases hepatic fatty acid oxidative capacity in the absence of AMPK activation in a rodent model of fatty liver disease [84]. Additional insight into the role of AMPK as a probable target for increasing mitochondrial biogenesis and fatty acid oxidative capacity is needed. Collectively, while limited examinations appear promising, targeting molecules involved in increasing

hepatic fatty acid oxidation to circumvent primary defects in mitochondrial dysfunction are warranted.

10. CONCLUSIONS

In summary, recent discoveries in molecular genetics have given us a better understanding of mitochondrial disease, and advances in gene and protein transfer technology offers the potential of finding effective therapies for these primary disorders. Limitations still exists in these strategies, including transduction efficiency, capacity, toxicity, duration and control of expression as well as high volume, low cost production. However, several aspects of the TAT-MTS fusion proteins are particularly appealing for treatment of mitochondrial diseases. Future investigations also should focus on treating individuals heterozygous for mitochondrial dysfunction and impaired hepatic fatty acid oxidation. Pharmacologic agents offer promising results in the ability to stimulate mitochondrial fatty acid oxidation and reduce hepatic steatosis and combination therapies could prove to be more effective for the treatment and prevention of mitochondrial disease.

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Figure 1. Biochemical pathway of mitochondrial β -oxidation. The four reactions of the pathway are shown. Bars indicate the blockage of reactions as a result of deficiency of LCHAD or mitochondrial trifunctional protein (MTP).

Figure 2. Histopathologic analyses. Representative liver sections from MTP wild-type ($MTPa^{+/+}$; A and C) and heterozygote ($MTPa^{+/-}$; B and D) mice stained with hematoxylin-eosin (A and B) and Oil-Red O (C and D). Original magnification $\times 20$. Reproduced with permission from Gastroenterology 2005;128:1381-1390 [4].

Figure 3.

Representative electron micrograph of hepatocytes from control wild-type mice ($MTPa^{+/+}$) (a) and mice heterozygous for a mitochondrial trifunctional protein defect ($MTPa^{+/-}$) (b) at 11700 $\times$ magnification. The $MTPa^{+/-}$ mice develop hepatic steatosis (see Figure 2). The mitochondria from the $MTPa^{+/-}$ mice were swollen with hypodense matrix and disrupted cristae. Reproduced with permission from *Gastroenterology*. 2005;128:1381–1390 [4].

Figure 4.

The fusion protein, TAT-sMtCK-DsRed.T3, was applied to NIH 3T3 cells in culture and then imaged by confocal microscopy. Panel A: CMX-Green. Panel B: TAT-sMtCK-DsRed.T3. Panel C: Overlay of Panels A and B.