Regulation of Mitochondrial Trifunctional Protein Modulates Nonalcoholic Fatty Liver Disease in Mice

Fatiha Nassir,1,2,4 Justin J. Arndt,1 Sarah A. Johnson1,4 and Jamal A. Ibdah,1,2,3,4

1Departments of Medicine-Division of Gastroenterology and Hepatology, 2Nutrition and Exercise Physiology, and 3Medical Pharmacology & Physiology, University of Missouri, Columbia, MO; 4United States Research Service, Harry S Truman Memorial VA Hospital, Columbia, MO.

Running Title: MTP, Mitochondria, and NAFLD

Conflict of interest: The authors declare no conflict of interest

Correspondence to: Jamal A. Ibdah, MD, PhD, Division of Gastroenterology and Hepatology, School of Medicine, University of Missouri, One Hospital Drive, DC043.00, CE405 Columbia, MO 65212. E-mail: ibdahj@health.missouri.edu

Abbreviations: FAO: fatty acid oxidation; HFD: high fat diet; LCHAD: long chain 3-hydroxyacyl Co-A dehydrogenase; MTP: mitochondrial trifunctional protein; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NAS: NAFLD activity score.
Abstract

Mitochondrial trifunctional protein (MTP) plays a critical role in the oxidation of long-chain fatty acids. We previously reported that aging mice (>9-month old) heterozygous for an MTP defect (MTP+/−) develop nonalcoholic fatty liver disease (NAFLD). We tested whether high-fat diet (HFD) accelerates NAFLD in young MTP+/− mice, and whether overexpression of the NAD+−dependent deacetylase SIRT3 deacetylates MTP and improves mitochondrial function and NAFLD. Three-month-old WT and MTP+/− mice were fed HFD (60% cal fat) for 16-weeks and livers were assessed for fatty acid oxidation (FAO) and NAFLD. Compared to wild type (WT), MTP+/− mice displayed reduced hepatic SIRT3 levels, reduced FAO, with increased hepatic steatosis and the inflammatory marker CD68. Hepatic overexpression of SIRT3 in HFD-fed MTP+/− mice increased hepatic MTP protein levels at the post-transcriptional level. Immunoprecipitation of MTP from liver mitochondria followed by Western blot with acetyl-lysine antibody showed higher acetylation of MTP in MTP+/− compared to WT mice. Overexpression of SIRT3 in MTP+/− mice significantly reduced the acetylation of MTP compared to β-gal controls, increased mitochondrial FAO and reduced hepatic steatosis, CD68, and serum ALT levels. Taken together, our data indicate that deacetylation of MTP by SIRT3 improves mitochondrial function and rescues NAFLD in MTP+/− mice.

Key words: Nonalcoholic fatty liver disease; SIRT3; Acetylation; Mitochondrial trifunctional protein; Mitochondria.
Introduction

Mitochondrial trifunctional protein (MTP) is a multienzyme complex composed of 4 α and 4 β subunits that catalyzes the last three steps in the β-oxidation of long-chain fatty acids. The α-subunit contains the activities of the long-chain enoyl-CoA hydratase, and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), while the β-subunit contains the activity of the long-chain thiolase (1). HADHA and HADHB are the genes encoding the α- and β-subunits, respectively. Human defects in MTP are recessively inherited and manifest as Reye-like syndrome and sudden infant death. Previously, we generated a knockout mouse model for MTP α-subunit null allele causing complete MTP deficiency (2). Similar to the human deficiency, mice homozygous for the MTP defect develop hepatic steatosis and suffer sudden neonatal death (2). In subsequent work, we have demonstrated that aging (>9-month old) heterozygous (MTP+/-) mice develop nonalcoholic fatty liver disease (NAFLD) on chow diet concomitant with reduced mitochondrial fatty acid oxidation (FAO) (3-5).

NAFLD is a spectrum that includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), and may progress to cirrhosis and hepatocellular carcinoma (6-8). NAFLD is strongly associated with obesity, insulin resistance and enhanced risk of cardiovascular disease (9, 10). Currently, NAFLD is the most prevalent form of liver disease in both children and adults, and is the primary cause of liver failure. Apart from life style modifications such as calorie restriction and exercise, no effective pharmacological treatment currently exists to treat NAFLD (7, 11). This is mainly due to lack of a clear understanding of the underlying pathophysiology of the disease.

Strong evidence supports a crucial role for the mitochondria in the pathophysiology of NAFLD (3, 5, 10, 12, 13). Mitochondrial dysfunction is a common finding in NAFLD patients and in animal models of NAFLD where ultrastructural abnormalities of liver mitochondria, reduced fatty acid oxidation (FAO), impaired hepatic ATP synthesis, reduced respiration and increased oxidative stress have been observed (2, 5, 12-17).
Sirtuins (SIRT) are a family of seven proteins (SIRT1-SIRT7) three of which are localized in the mitochondria (SIRT3-5). SIRT3 is a NAD+-dependent deacetylase that regulates the function of many mitochondrial proteins and may play a protective role against NAFLD (18-20). SIRT3 deficient mice fed a chronic high-fat diet develop accelerated obesity, insulin resistance, and steatohepatitis, compared to wild type (WT) mice (21). Among the mitochondrial sirtuins, SIRT3 possesses the most robust deacetylase activity (22). SIRT3 deficient mice show increased hyperacetylation of mitochondrial proteins and reduced FAO compared to wild type mice (23, 24). Label-free quantitative mass spectrometry analysis of the lysine acetylome from SIRT3 deficient mice identified MTP α-subunit as a highly acetylated protein (24). It is not known whether SIRT3 gain of function rescues NAFLD, particularly in our mouse model of mitochondrial dysfunction.

Herein, we propose that regulation of MTP modulates NAFLD. MTP plays a central role in FAO and hence factors that increase its activity are likely to rescue NAFLD by improving FAO. However, little is known about MTP regulation. In this study, we tested whether SIRT3 regulates MTP via deacetylation and whether deacetylation of MTP is an underlying mechanism in the rescue of NAFLD in our mouse model. We utilized high fat diet in young MTP+/− mice to accelerate development of NAFLD. In addition, we overexpressed SIRT3 in the liver of these mice to determine whether deacetylation of MTP is an underlying mechanism in the regulation of MTP and NAFLD.

Methods

Animals, diet and adenoviral injection

The animal protocol was approved by the institutional Animal Care and Use Committee at the University of Missouri-Columbia. Male mice on C57BL/6 background were used in these studies (2). WT and MTP+/− mice genotype was determined by PCR using primers that differentiate the mutant from the WT allele, as described previously (2). Mice were housed in temperature-controlled rooms (21°C) with a 06.00–18.00 h light: 18.00–06.00 h dark cycles that were maintained throughout the experimental period. Mice were given...
standard (chow) diet (Formulab 5008, Purina Mills, St. Louis, MO, USA) or HFD (D12492, Research Diets INC, 60% cal fat). For HFD experiments, animals (three-month old) were given HFD for 16 weeks after which they were sacrificed. For SIRT3 studies, three-month old WT and MTP+/− mice were given HFD for 16 weeks after which adenoviral expression of β-gal (control) or SIRT3 (abm, Richmond, BC) was performed (9×10⁹ virus particles/g body weight, diluted in saline) via tail vein injection of the adenovirus and the animals were studied 3 days post-injection. The Virus was amplified and purified using Add-N-Pure Adenovirus Purification Kit (abm, Richmond, BC) as described by the manufacturer. All mice were fasted overnight, anesthetized with sodium pentobarbital (50mg/kg) and blood and tissues were collected. Livers and other tissues were frozen in liquid nitrogen for biochemical analysis and western blot analysis or stored in RNA-later solution for gene expression studies. Tissue samples were also fixed using 10% neutral buffered formalin for histological analysis, or in Tissue-Tec O.C.T to prepare frozen sections.

Isolation of mitochondria and measurement of fatty acid oxidation

Mitochondria were prepared from liver samples as described previously (25) and according to modified method of Koves et al (26). The oxidation of fatty acids in the liver and isolated mitochondria was measured using ¹⁴C-Palmitate (American Radiochemical) in fresh tissues and isolated liver mitochondria as described previously (27). The ¹⁴CO₂ resulting from complete combustion of ¹⁴C-Palmitate was counted using a liquid scintillation counter.

Western blotting

Homogenates from liver or mitochondria were separated on 4-20% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with specific antibodies to MTP, Acetyl-Lysine, SIRT1 and SIRT3 (Cell Signaling), SIRT5 (Abcam), SIRT4, SIRT7, tubulin (Santa Cruz), SIRT6 (MyBioSource, San Diego, CA). Proteins were visualized with the molecular imager ChemiDOC XRS⁺ and the intensities of the bands were quantified using Quantity One Analysis Software (Bio-Rad).
RNA extraction and RT PCR

Total RNA was extracted from liver samples using RNeasy Mini Kit (Qiagen) and subjected to cDNA Reverse Transcription (Applied Biosystems) and RT quantitative PCR using Power SYBR Green PCR Mix on StepOnePlus Real-Time PCR System (Applied Biosystems). The data were analyzed using the StepOne software using specific real-time PCR primers. The real time primers for HADHA were purchased from Bio-Rad.

Acetylation of MTPα

Mitochondria were isolated from liver tissue as described above and mitochondrial pellets were re-suspended in lysis buffer containing protease inhibitors. MTPα was immunoprecipitated from mitochondrial lysate using anti-MTPα antibody cross linked to protein A/G agarose beads. The crosslinking of the antibody to protein A/G agarose beads and the immunoprecipitation of MTPα was conducted as described in the Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific). Equal amount of immunoprecipitated proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane which was then probed either with anti-MTPα antibody to determine the mitochondrial level of MTPα or with anti-acetyl lysine to determine its acetylation level.

Hepatic lipid content and histology

Hepatic lipids were extracted using chloroform:methanol (2:1, v/v) and hepatic triglyceride content was analyzed using triglyceride assay kit (Wako diagnostics, Mountain View, CA). Oil-Red O staining for neutral lipids was determined using liver frozen sections. Staining of liver sections for hematoxylin and eosin (H&E), and CD68 were performed using formalin fixed liver sections.

Statistics.

Statistical significance was determined with student t-test. Data are expressed as mean ± SE; a P value of less than 0.05 was considered statistically significant.
Results

**HFD accelerates NAFLD in MTP<sup>+/−</sup> mice.** All mice gained ~10 g in body weight on HFD but no differences in weight gain, weekly food intake, or body and liver weight were observed at the end of the 16-week HFD between the WT and MTP<sup>+/−</sup> mice (Table 1). MTP<sup>+/−</sup> mice on HFD had 2-fold increase in hepatic lipid accumulation compared to WT mice (Figure 1A). This was confirmed by Oil Red O (ORO) staining for neutral lipids and H&E staining (Figure 1B) of liver sections. The increase in hepatic triglyceride in MTP<sup>+/−</sup> mice was associated with >50% reduction in FAO compared to WT (Figure 1C). MTP<sup>+/−</sup> mice also displayed increased levels of serum ALT (Figure 1D) and CD68 (Figure 1E) compared to WT.

**SIRT3 levels are reduced in MTP<sup>+/−</sup> mice.** We examined the abundance of the different sirtuins in WT livers and found that SIRT3 is the highly expressed sirtuin in this organ (Figure 2A). Importantly, hepatic SIRT3 levels were reduced in MTP<sup>+/−</sup> mice compared to WT (Figure 2B, C), and this was concomitant with higher acetylation of MTP in the livers of MTP<sup>+/−</sup> compared to WT mice (Figure 2D).

**Overexpression of SIRT3 reduced hepatic triglyceride in MTP<sup>+/−</sup> mice.** We used mouse SIRT3 adenovirus to specifically overexpress SIRT3 in the liver of WT and MTP<sup>+/−</sup> mice. SIRT3 overexpression increased hepatic SIRT3 levels in both WT and MTP<sup>+/−</sup> livers (Figure 3A, B) and reduced ALT levels minimally in WT and dramatically in MTP<sup>+/−</sup> compared to β-gal injected mice (Figure 3C). Hepatic triglyceride levels were higher in MTP<sup>+/−</sup> compared to WT β-gal mice (Figure 3D). Overexpression of SIRT3 reduced triglyceride levels in MTP<sup>+/−</sup> but not in WT livers (Figure 3D). Oil-Red O and H&E staining confirmed the reduction in hepatic triglyceride in MTP<sup>+/−</sup> mice upon SIRT3 overexpression (Figure E). SIRT3 overexpression also reduced the inflammatory marker CD68 in MTP<sup>+/−</sup> livers (Figure 3F).

**SIRT3 deacetylates MTP and restores its levels in MTP<sup>+/−</sup> mice.** We examined hepatic MTP levels in WT and MTP<sup>+/−</sup> mice injected with control β-gal or SIRT3 adenovirus. As expected, the MTP protein (Figure 4A, B) and mRNA levels (Figure 4C) were about 50% lower in the MTP<sup>+/−</sup> compared to WT - β-gal injected mice. SIRT3 overexpression restored hepatic MTP protein levels to WT levels (Figure 4A, B).
without an increase in MTP gene expression (Figure 4C) suggesting that SIRT3 regulates MTP levels at the post-transcriptional level. The overexpression of SIRT3 in MTP<sup>+/−</sup> mice was specific to the liver since no change was observed in other tissues (Figure 4D). SIRT3 overexpression also had no effect on body and liver weight (Table 2). To determine whether SIRT3 deacetylates MTPα, we subjected liver mitochondrial lysates from MTP<sup>+/−</sup> mice injected with control or SIRT3 adenovirus, to immunoprecipitation with anti-MTPα antibody. Equal amounts of MTPα immunoprecipitates were subjected to western blot with either MTPα or acetyl lysine (Ac-Lys). As expected, Figure 4E-I shows equal amounts of MTPα protein immunoprecipitated from both β-gal and SIRT3 overexpressing mice. However, western blot with Ac-Lys antibody shows significantly lower acetylation of MTPα in the SIRT3 compared to β-gal controls (Figure 4E-II). These data document in vivo deacetylation of MTP by SIRT3.

**SIRT3 overexpression improves mitochondrial function in MTP<sup>+/−</sup> mice.** Mitochondrial function was evaluated in SIRT3 and β-gal injected MTP<sup>+/−</sup> mice. FAO was increased in both liver tissue (Figure 5A-I) and in isolated mitochondria (Figure 5A-II) from mice overexpressing SIRT3 compared to β-gal controls. Blood levels of β-hydroxybutyrate (β-HB) were also increased in SIRT3 overexpressing livers as compared to β-gal controls (Figure 5A-III), consistent with increased FAO. The improvement in hepatic mitochondrial function in mice overexpressing SIRT3 were due to the specific increase in the hepatic level of SIRT3 without significant changes in the protein levels of other sirtuins (Figure 5B-I,II).

**Discussion**

MTP is a multienzyme protein complex that plays a major role in mitochondrial FAO as it carries three of the four enzymes required for long chain FAO. We previously reported that MTP deficiency in mice results in hepatic steatosis, cardiomyopathy, and neonatal sudden death (2), while aging heterozygous MTP<sup>+/−</sup> mice develop hepatic steatosis and insulin resistance (3, 5). In the current study, we show that HFD accelerates NAFLD in the susceptible young MTP<sup>+/−</sup> mice, which was associated with highly
acetylated MTP α-subunit, and reduced FAO. We have shown that hepatic overexpression of SIRT3 reduces MTP acetylation levels, restores FAO, and improves NAFLD in mice.

MTP catalyzes three consecutive steps in the mitochondrial β-oxidation of long-chain acyl-CoA esters: 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. MTP enzymatic deficiencies are important causes of human disease. Formation of a protein complex between α and β subunits is important for MTP stability. This is supported by the observation that individual α and β subunits are unstable when expressed heterologously (28). Genes for the α and β subunits are adjacent to each other in a head-to-head configuration on chromosome 2p23, and are transcribed from the same bidirectional promoter region (29); hence expression of the two subunits may be coordinated. A unique feature is the association between MTP defects and acute fatty liver of pregnancy (30). Mothers carrying fetuses with 3-hydroxyacyl-CoA dehydrogenase deficiency, develop a life-threatening condition of acute fatty liver of pregnancy highlighting the important role of MTP in development of fatty liver disease.

Given the key role of MTP in fatty acid oxidation and its significance to human disease, understanding of MTP regulation is of paramount importance.

In the present study, we document that HFD accelerates NAFLD in the susceptible young MTP+/− mice, which was associated with reduced SIRT3 levels, highly acetylated MTP α-subunit, and reduced FAO. Reduced SIRT3 levels were also observed in aging MTP+/− mice (data not shown). Interestingly, hepatic SIRT3 was also reported to be reduced in mouse models of NAFLD and in NASH patients compared to control subjects (23, 31) suggesting a significant role for SIRT3 in NAFLD. We show that hepatic overexpression of SIRT3 improved NAFLD in the MTP+/− mouse model but had no effect on lipid accumulation in WT mice. This is consistent with reports that overexpression of SIRT3 in C57B6 mice liver in vivo did not protect against lipid accumulation induced by HFD while SIRT3 deficient mice fed a HFD show accelerated obesity, insulin resistance, hyperlipidemia, and steatohepatitis compared to wild-type (WT) mice (21, 32). The
observed beneficial effect of SIRT3 overexpression in the MTP +/- mouse model is intriguing and may suggest that MTP +/- mice benefited the most from SIRT3 overexpression due to the higher MTP acetylation status and lower hepatic SIRT3 levels in these mice. The reduced SIRT3 level in this mouse model warrants additional studies to explore the underlying mechanisms. It is possible that knockdown of the MTP gene downregulates SIRT3 levels. We have documented in an earlier study that MTPalpha +/- mice display enhanced extramitochondrial fatty acid oxidation with higher reactive oxygen species generation compared to the WT mice (3). Several recent studies have reported an attenuated SIRT3 expression in response to enhanced reactive oxygen species generation (33, 34).

Our data document that overexpression of SIRT3 in the mouse liver reduces the acetylation of MTP and increases hepatic MTP protein levels without alterations in HADHA gene expression suggesting that SIRT3 regulates MTP at the post-transcriptional level. The reduced MTP acetylation and the improvement in the oxidation of fatty acids in the mitochondria of MTPalpha +/- mice upon SIRT3 overexpression was independent from changes in the hepatic levels of other sirtuins indicating a direct role of SIRT3 in the regulation of MTP. Thus, SIRT3 may enhance FAO and mitochondrial function through deacetylation and increased stability of MTP. The mechanism by which lysine acetylation causes unstable MTP complex is unknown and requires future studies to investigate how SIRT3 specifically enhances MTP stability.

In summary, we show that SIRT3 regulates hepatic levels of MTP by modulating its acetylation status, which in turn leads to regulation of mitochondrial FAO impacting development and rescue of NAFLD.

Support: This work was funded by institutional funds from University of Missouri-Columbia (JA Ibdah) and the University of Missouri Research Board grant (F. Nassir).
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Table 1. Effect of HFD on food intake, weight gain, body and liver weight in WT and MTP<sup>−/−</sup> mice

| Mice                      | WT          | MTP<sup>−/−</sup> |
|---------------------------|-------------|-------------------|
| Average weekly food intake (g) | 15.7±0.02   | 16.8±0.03         |
| Average weight gain (g)    | 12.0±0.9    | 11.1±1.0          |
| Body weight (g)            | 43.5±1.4    | 41.2±0.8          |
| Liver weight (g)           | 2.0±0.3     | 1.8±0.3           |

Data are expressed as means ± SEM of n=10, no statistical difference, p>0.5 using t-test.
Table 2. Effect of SIRT3 injection on body and liver weight

| Mice          | MTP^{+/-}-β-gal | MTP^{+/-}-SIRT3 |
|---------------|-----------------|-----------------|
| Body weight (g) | 41.9 ± 1.4      | 42.7 ± 1.2      |
| Liver weight (g) | 1.76 ± 0.2      | 1.78 ± 0.1      |

Data are expressed as means ± SEM of n=10, no statistical difference, p>0.5 using t-test
FIGURES

Figure 1. HFD accelerates hepatic steatosis and liver inflammation in young MTP<sup>+/−</sup>. A) Hepatic triglyceride. B) Oil-red-O and H&E staining of liver sections. C) Fatty acid oxidation in mitochondria from WT and MTP<sup>+/−</sup> mice. D) Serum ALT. E) CD68 staining of liver sections. Data are mean ± SE of n=6, *P<0.05 using T-test.
Figure 2. SIRT3 levels are reduced in MTP+/- mice. A) Hepatic expression of the different sirtuins in WT mice. B) Hepatic levels of SIRT3 in young WT and MTP+/- mice fed HFD. D) HADHA was immunoprecipitated from mitochondrial lysate from WT and MTP+/- mice and equal amount of immunoprecipitated proteins were probed for Ac-lys. Data are mean ± SE of n=3-6.
Figure 3. Overexpression SIRT3 improves NAFLD in MTP<sup>−/−</sup> mice. A) Overexpression of SIRT3 in WT and MTP<sup>−/−</sup> mice. B) Quantification of hepatic SIRT3 levels in WT type and MTP<sup>−/−</sup> mice injected with β-gal or SIRT3 adenovirus. C) ALT levels. D) Hepatic triglyceride. E) Oil-red-O and H&E staining of liver sections from MTP<sup>−/−</sup> mice injected with β-gal or SIRT3 adenovirus. F) Staining of liver sections from MTP<sup>−/−</sup> mice injected with β-gal or SIRT3 adenovirus. Data are mean ± SE of n=3-6. (#: difference between WT β-gal and WT SIRT3. *: difference between WT-β-gal and MTP<sup>−/−</sup>-β-gal and #: difference between MTP<sup>−/−</sup>-β-gal and MTP<sup>−/−</sup>- SIRT3.
Figure 4. SIRT3 upregulates MTP in MTP$^{+/−}$ mice. A) Hepatic levels of HADHA in WT and MTP$^{+/−}$ mice expressing β-gal and SIRT3. B) Quantification of HADHA levels. C) Hepatic HADHA mRNA level. D) Specific overexpression of SIRT3 in the liver; white bars (MTP$^{+/-}$-β-gal) and grey bars (MTP$^{+/-}$-SIRT3). E) Immunoprecipitation (IP) of HADHA from liver mitochondrial lysates from mice injected with β-gal or SIRT3 adenovirus followed by western blot (WB) for HADHA (I) or for acetyl-lysine to detect acetylated MTPα (Ac-MTPα) (II) (P: pool of mitochondrial lysate; na: no antibody). Data are mean ± SE of n=3-4. *: difference between WT-β-gal and MTP$^{+/-}$-β-gal and &: difference between MTP$^{+/-}$-β-gal and MTP$^{+/-}$-SIRT3.
Figure 5. Adenoviral overexpression of SIRT3 in vivo restores FAO in young MTP+/− mice on HFD. A) Complete oxidation of 14C-radiolabeled palmitate to CO2 in liver tissue (I) and isolated mitochondria (II), and serum levels of β-Hydroxybutyrate (β-HB) (III). B) Hepatic levels of the different sirtuins in the liver from mice injected with β-gal or SIRT3 adenovirus (I) and quantification (II). Data are mean ± SE of n= 5.