Mice Heterozygous for a Defect in Mitochondrial Trifunctional Protein Develop Hepatic Steatosis and Insulin Resistance

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Background & Aims: **Little is known about the role of mitochondrial β-oxidation in development of nonalcoholic fatty liver disease (NAFLD). Mitochondrial trifunctional protein (MTP) catalyzes long-chain fatty acid oxidation. Recently, we generated a mouse model for MTP deficiency and reported that homozygous (*MTPa*$^{-/-}$) mice suffer neonatal death. In this study, we investigated effects of heterozygosity for the MTP defect on hepatic oxidative stress, insulin resistance, and development of NAFLD in mice.** ***Methods:*** **We evaluated liver histopathology, serum alanine aminotransferase (ALT), glucose, fatty acids, and insulin levels in *MTPa*$^{+/-}$ and *MTPa*$^{+/+}$ littermates. Insulin resistance was evaluated using glucose tolerance test (GTT) and insulin tolerance test (ITT). Liver tissues were used to measure triglyceride and fatty acid content, activity of superoxide dismutases (SOD) and glutathione peroxidase (GPx), glutathione (GSH), and cytochrome P-450 2E1 expression.** ***Results:*** **Aging but not young *MTPa*$^{+/-}$ mice developed hepatic steatosis with elevated ALT, basal hyperinsulinemia, and increased insulin area under curve (AUC) on GTT compared with *MTPa*$^{+/+}$ littermates. In response to insulin challenge, aging *MTPa*$^{+/-}$ mice had slower rate of glucose disappearance and increased glucose AUC. Significant hepatic steatosis and insulin resistance developed concomitantly in the *MTPa*$^{+/-}$ mice at 9–10 months of age. Aging *MTPa*$^{+/-}$ mice had higher antioxidant activity of total SOD and GPx, lower GSH, and increased expression of cytochrome P-450 2E1, consistent with increased hepatic oxidative stress.** ***Conclusions:*** **Heterozygosity for β-oxidation defects predisposes to NAFLD and insulin resistance in aging mice. Impairment of mitochondrial β-oxidation may play an important role in pathogenesis of NAFLD.**

Nonalcoholic fatty liver disease (NAFLD) is a common clinicopathologic syndrome that encompasses a broad spectrum, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), and progresses to cirrhosis in a significant number of patients.[1,2] The prevalence of NAFLD is approximately 20% in the general population, making this condition the most common liver disease in the United States.[3] NAFLD is most commonly diagnosed in adults aged 45–55 years but can also affect children.[4] Although NAFLD is strongly associated with insulin resistance,[1–3] its pathogenesis is incompletely understood, and little is known about the role of alterations in mitochondrial fatty acid metabolism in development of NAFLD. Mitochondrial β-oxidation of fatty acids is the major source of energy for the skeletal muscles and heart and plays an important role in the intermediary metabolism in the liver. Mitochondrial trifunctional protein (MTP) is a heterooctamer that consists of 4 α- and 4 β-subunits. The α-subunit contains both the long-chain hydratase and 3-hydroxyacy-CoA dehydrogenase (LCHAD) functions that catalyze the second and third steps, respectively, of the β-oxidation spiral. The β-subunit contains the long-chain thiolase activity that catalyzes the fourth step in the β-oxidation spiral.[5,6] The human cDNAs encoding both α- and β-subunits have been isolated and characterized.[7,8] Both subunit genes, HADHA and HADHB, have been localized to chromosome 2p23.[9]

Human MTP defects are recessively inherited and may cause deficiency of 1 or all 3 enzymatic activities in the MTP complex.[5,6,8,10–13] The majority of children with MTP defects present with hepatic dysfunction associated with predominantly microvesicular hepatic steatosis that resembles Reye syndrome.[13] In addition, we and others have documented that women who carry fetuses with MTP defects often develop acute fatty liver of pregnancy

Abbreviations used in this paper: AUC, area under curve; GPx, glutathione peroxidase; GSH, glutathione; GTT, glucose tolerance test; ITT, insulin tolerance test; LCHAD, long chain 3-hydroxyacyl-CoA dehydrogenase; MTP, mitochondrial trifunctional protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ROS, reactive oxygen species; SOD, superoxide dismutase.

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and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome.[13–15]

Recently, we reported the development of a mouse model with an MTP α-subunit null allele.[16] The homozygous ($MTPa^{-/-}$) mice lack both MTP α- and β-subunits, have biochemical changes identical to those of human deficiency, and suffer sudden death 6–36 hours after birth. Analysis of the histopathologic changes in the $MTPa^{-/-}$ pups revealed rapid development of hepatic steatosis after birth, followed by acute degeneration and necrosis of the cardiac and diaphragmatic myocytes.[16]

The purpose of this study was to assess the phenotype in the heterozygous ($MTPa^{+/-}$) mice. We investigated whether heterozygosity for the MTP defect predisposes to development of adult or pediatric NAFLD in mice. We also assessed the $MTPa^{+/-}$ mice for alterations in insulin sensitivity, hepatic fatty acids content, and oxidative stress.

Materials and Methods

Mice

A knockout mouse model with MTP α-subunit null allele was generated as reported earlier.[16] Mice were maintained at Wake Forest University School of Medicine Transgenic Mouse Core Facility. All mice used in this study were male littermates maintained on standard ad libitum chow diet (Agway Prolab 3000; Agway Country Foods, Syracuse, NY) comprising 22% protein, 5% fat, and 53% carbohydrate (the remaining 20% consists of crude, fiber, and moisture). The care of the animals was in accordance with Wake Forest University School of Medicine and Institutional Animal Care and Use Committee guidelines. The mouse genotype was determined by PCR using primers that distinguish the mutant allele from the wild-type allele as described previously.[16] All PCR analyses were confirmed in duplicates.

Histopathologic Analysis

For routine histology, liver tissues were collected and fixed in cold 4% paraformaldehyde for 24 hours at 4°C, followed by postfixation in 70% ethanol. Tissues were then processed into paraffin blocks, sectioned at 4 μm, and stained with H&E.[16] Tissues used to detect the presence of fat were quick-frozen, sectioned at 4 μm, and stained using oil red O (Hartman-Leddon Co., Philadelphia, PA). For the assessment of hepatic steatosis, histopathologic criteria proposed by Brunt et al[17] were adopted. Hepatic steatosis was graded as follows: absent (score 0); or present in <one third of hepatocytes (score 1); in one third to two thirds of hepatocytes (score 2); or in >two thirds of hepatocytes (score 3). A board-certified veterinary pathologist blinded to the experimental group (J.M.C.) reviewed all sections. For electron microscopy, tissues were collected and fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, embedded in Spurr's resin, sectioned at 90 nm, and stained with uranyl acetate/lead citrate. Electron microscopy was performed at Wake Forest University School of Medicine Electron Microscopy Core Facility using Phillips 400 TEM electron microscope (Phillips, Holland) at ×2500–22,000 magnification.

Biochemical Analysis

Cardiac puncture was used to obtain blood samples at time of death. Sera were analyzed using an automated analyzer (Technicon CHEM I; Bayer Corp., Tarrytown, NY) for measurement of glucose and alanine aminotransferase (ALT). Insulin was measured in the sera using Ultrasensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL) according to the manufacturer's recommended protocol. Liver tissues were stored frozen at −80°C prior to analysis. For measurement of liver triglyceride content, lipids were extracted from approximately 100–200 mg minced liver by the chloroform/methanol method, and the total triglyceride was determined colorimetrically as described previously,[18] using enzymatic assay reagents purchased from Roche Diagnostics (Triglycerides/GB, Cat. 450032). C8-C18 fatty acids in liver homogenates and C8-C26 fatty acids in plasma were analyzed using capillary gas chromatography-mass spectrometry as previously described.[19] Serum-free fatty acids (FFA) were measured using NEFA-C Kit (Wako Chemicals USA, Inc., Richmond, VA) according to the manufacturer's recommended protocol. Hepatic oxidative stress was evaluated by measurement of the total superoxide dismutases (SOD) activity, cellular glutathione peroxidase (GPx) activity, and glutathione (GSH) levels in the liver. SOD activity was measured in liver homogenate using spectrophotometric assay that is independent of the type of SOD (such as Cu/Zn-SOD, Mn-SOD, or Fe-SOD) using a kit (Bioxytech SOD-525 Assay Kit, Oxis Research, Portland, OR) according to the manufacturer's recommended protocol. GPx activity and GSH level in liver homogenates were measured using colorimetric assays (Bioxytech GPx-340 Assay and Bioxytech GSH-400 Assay, respectively, Oxis Research) according to the manufacturer's recommended protocol.

Evaluation of Cytochrome P450 2E1 Protein Expression

Immunoprecipitation and immunoblotting analyses were used for detection and quantitative measurement of cytochrome P-450 2E1 expression. Frozen liver tissues were homogenized and prepared for immunoprecipitation according to a standard protocol. Tissue extracts containing equal protein amounts (~300 μg) were precleared using protein A/G PLUS-agarose beads and 1.0 μg normal goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) followed by immunoprecipitation using protein A/G PLUS-agarose beads and Anti-Rat CYP2E1 raised in goat (Daiichi Pure Chemicals Co., Tokyo, Japan), according to the recommended protocol. The immunoprecipitated proteins were resolved using 10% Acrylamide Bis gel (Amresco, Solon, OH) at 100 V. Proteins were then transferred to Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom) at 100 V at room temperature

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