Molecular Genetics and Metabolism 110 (2013) 248–254

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Biotinidase knockout mice show cellular energy deficit and altered carbon metabolism gene expression similar to that of nutritional biotin deprivation: Clues for the pathogenesis in the human inherited disorder

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ARTICLE INFO

Article history:
Received 8 August 2013
Received in revised form 30 August 2013
Accepted 30 August 2013
Available online 8 September 2013

Keywords:
Biotin deficiency
Biotinidase knockout
energetic deficit
AMPK
Insulin sIgnaling pathway
Insulin sensitivity

ABSTRACT

Biotin is the prosthetic group of carboxylases that have important roles in the metabolism of glucose, fatty acids and amino acids. Biotinidase has a key role in the reutilization of the biotin, catalyzing the hydrolysis of biocytin (ε-N-biotinyl-L-lysine) and biocytin-containing peptides derived from carboxylase turnover, thus contributing substantially to the bioavailability of this vitamin. Deficient activity of biotinidase causes late-onset multiple carboxylase in humans, whose pathogenic mechanisms are poorly understood. Here we show that a knock-out biotinidase-deficient mouse from a C57BL/6 background that was fed a low biotin diet develops severe ATP deficit with activation of the energy sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK), inhibition of the signaling protein mTOR, driver of protein synthesis and growth, and affecting the expression of central-carbon metabolism genes. In addition, sensitivity to insulin is augmented. These changes are similar to those observed in nutritionally biotin-starved rats. These findings further our understanding of the pathogenesis of human biotinidase deficiency.

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1. Introduction

Biotin is the prosthetic group of the carboxylase enzymes, propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC) 3-methylcrotonyl-CoA carboxylase (MCC) and acetyl-CoA carboxylase (ACC) that have important roles in tricarboxylic acid cycle anaplerosis, gluconeogenesis, fatty acid synthesis and amino acid and odd-chain fatty acid catabolism. Biotinidase has a key role in the reutilization of the biotin contributing substantially to the bioavailability of this vitamin [1]. Biotinidase catalyzes the hydrolysis of biocytin (ε-N-biotinyl-L-lysine) and biocytin-containing peptides derived from carboxylases turnover, thereby recycling biotin. Deficient activity of biotinidase causes late-onset multiple carboxylase in humans (MIM 253260). Biotinidase deficiency results in the inability to reutilize biotin; however the pathogenic mechanisms of the disorder are poorly understood [2]. Clinical features of biotinidase deficiency include neurological features, such as hypotonia, seizures, respiratory problems, ataxia, hearing loss, and vision problems, cutaneous problems, such as eczematous skin rash, alopecia, conjunctivitis, and candidiasis, and metabolic abnormalities, including ketolactic acidosis, organic aciduria, and mild hyperammonemia. If untreated, symptoms usually became progressively worse, possibly resulting in coma and death [3].

We have studied various aspects of biotin deprivation in the biotin-deficient rat (*Rattus norvegicus*) liver, the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae*, supplied with abundant glucose provision, and discovered paradoxical changes in the expression of central-carbon metabolism genes [4]. In addition, we found deficits in ATP [5], severe mitochondrial dysfunction and an increase in the sensitivity to insulin in rats nutritionally deprived of biotin [6].

A knock-out (KO) biotinidase-deficient mouse (C57BL/6 background) with a null mutation was developed that results in no detectable serum biotinidase activity or cross-reacting material to antibody prepared against biotinidase [2]. When fed a biotin-deficient diet for 10 to 14 days, these mice developed neurological and cutaneous symptoms, carboxylase deficiency, mild hyperammonemia, and increased urinary excretion of 3-hydroxyisovaleric acid, biotin and biotin metabolites. The clinical features were reversed with biotin supplementation. These animals appear to be an excellent model for the enzyme-deficiency in humans.

We now demonstrate that mouse genetic biotinidase deficiency leads to genomic and metabolic changes similar to those produced in the biotin-starved rat. These studies provide new and important information that may be relevant to the untreated human disorder furthering our understanding of the pathogenesis of the human disorder [7].

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http://dx.doi.org/10.1016/j.ymgme.2013.08.018

2. Materials and methods

2.1. Design considerations

We compared various parameters between wild type (WT) mice and biotinidase-deficient, knock-out (KO) mice after both groups were fed a biotin-deficient diet for five days. At this time the weights of both groups of mice were not significantly different and the KO mice did not exhibit any clinical symptoms of biotinidase deficiency. We have previously demonstrated the importance of studying the early specific molecular and metabolic effects of biotin deficiency before other non-specific effects, such as malnutrition.

2.1.1. Animals

In these experiments we used mice of C57BL/6 background that are homozygous for the knocked-out biotinidase BTD gene (KO) [2]. Wild-type (WT) mice were of the same strain and homozygous for the normal BTD gene. The mice were maintained under standard housing conditions in a 12-h light–dark cycle with free access to food and water. Approval was obtained from the Animal Research Committee (CINVA) of the National Institute of Medical Sciences and the Nutrition “Salvador Zubirán” (INNSZ) for maintenance of breeding colonies and for all the procedures performed on mice in this project. Genotyping of biotinidase-deficient KO mice was performed as described previously [2].

2.1.2. Dietary manipulation

Standard breeder diet (2018S, Harlan Teklad, Madison, WI), supplemented with biotin (0.9 mg per kilogram), was fed to the animals for breeding. The pups were suckled by either wet nursing WT mice in the case of WT pups or KO dams for KO pups, until they were three weeks old. One week prior to weaning genotyping was performed. At the time of weaning the experiments were started by feeding both the KO and WT mice with a biotin-deficient diet (22.1% alcohol-extracted casein as the sole source of protein TD. 98161 from Harlan Teklad, Madison, WI) for five days.

2.2. Streptavidin Western blot analysis of holocarboxylases and signaling proteins

Aliquots of liver were homogenized with an extraction buffer containing HEPES 50 mM, KCl 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerol phosphate 5 mM, Triton X-100 0.1% (v/v), protease inhibitors (MiniComplete, Roche), sodium fluoride 50 mM, sodium orthovanadate 1 mM, sodium pyrophosphate 5 mM and PMSF 0.2 mM (all from Sigma-Aldrich). The homogenates were centrifuged at 12,000 ×g for 30 min at 4 °C and supernatants were used. Aliquots containing 100 or 50 μg of total protein were subjected to SDS-PAGE electrophoresis. The gel was transferred on a 0.45 μm nitrocellulose membrane, at 100 V for 60 min. To assess biotin status, we determined the degree of carboxylase biotinylation using streptavidin Western blot analysis and revealed with streptavidin–alkaline phosphatase for 30 min. For signaling proteins, the membranes were performed using various antibodies: 1:1000 AMPKα (23A3) #2603, phospho-AMPKα (Thr172) #2531, phospho-ACC1/2 (Ser79) #3661, phospho-Akt (Thr308) #135650, p-mTOR (Ser2448) #5536, as a secondary antibody, 1:2000 anti-rabbit IgG #7074S anti-mouse IgG #7076 (Cell Signaling Technology), αActin #A2103 (Sigma Aldrich) and α-Tubulin #sc-8035 (Santa Cruz Biotechnology). Visualization was made using as chemiluminescent substrate HRP Immobilon™ Western (Millipore Corporation, Billerica, MA), and was analyzed in a FUSION FX5 system (Vilber Lourmat, France). Total protein was determined by the Bradford method [8].

2.2.1. Acylcarnitine analysis

To further evaluate biotin status, dried blood spots were collected from all rats on Whatman 903 filter paper. Blood spots were analyzed using electrospray ionization liquid chromatography–mass spectrometry (LC–MS) with a Quattro Micro API (MicroMass) tandem mass spectrometer. All procedures for sample preparation and LC–MS analysis were performed by NeoGram AAAC spectrometry kit (PerkinElmer, MA, USA) according to the manufacturer's protocol. Briefly, single disks were punched from each dried blood spot using an automatic 3-mm punch. One disk was added per well. Using a multichannel pipette, 190 μL of extraction solution, containing a mixture of the respective stable isotope-labeled internal standards, was added to each well. The plate was covered with aluminum foil, followed by shaking at 650 ×g and incubation for 30 min at 30 °C. The plate was finally placed in the auto-sampler for testing.

2.2.2. Adenine nucleotides

ATP, ADP and AMP concentrations were determined by HPLC according to the method of Delaney and Geiger [9]. Mice were sacrificed by concussion. A portion of hepatic tissue (0.2–0.4 g) was removed and immediately immersed in 1 mL of perchloric acid 8%, homogenized, and centrifuged (10,000 ×g for 10 min at 4 °C). The supernatant was collected and immediately was neutralized with K_2CO_3 3 M (6% final volume) and recentrifuged. The adenine nucleotide content was determined by HPLC [7]. Briefly, 100 μL of a 1:10 dilution of each sample was analyzed by HPLC. Nucleotides were separated on an ACE 5 μm C18 (150 × 4.6 mm) column using an HPLC KNAUER system (Smartline System Isocratic HPLC with UV detection). The mobile phase consisted of KH_2PO_4 1 M, 20% of methanol and ION PAIR COCKTAIL Q6 4 mM (hexyltriethylammonium phosphate) with a flux of 1.5 mL/min. The external standard was treated identically to that of the hepatic homogenates; the ATP, ADP and AMP spikes were identified based on their retention times. The data are expressed in μmol/g of wet tissue.

2.2.3. Gene expression analyses

RNA was isolated from liver samples; cDNA was generated by M-MLV reverse transcriptase enzyme (Invitrogen) and random hexamers (deoxy-NTP6; Amersham Science). The analysis was performed by quantitative reverse-transcriptase PCR (qRT-PCR) on an ABI PRISM 7700 real time PCR machine (Applied Biosystems) using Taqman probes. The concentration of each target mRNA was determined and normalized to 18S rRNA using the $2^{-\Delta\Delta CT}$ method [10].

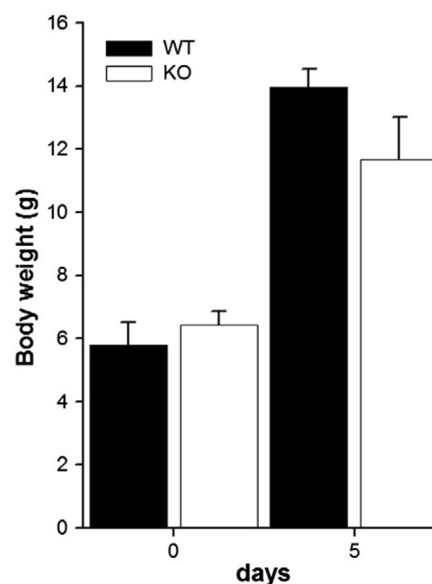

Fig. 1. Variation in body weight between WT mice (solid bars) and the KO mice (clear bars) is not statistically significant after being fed the biotin-deficient diet for five days. Five animals were included in each group. Values are the mean ± SD; n = 5.

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