Molecular Genetics and Metabolism Reports 2 (2015) 51–60

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Corrective effects of hepatotoxicity by hepatic Dyrk1a gene delivery in mice with intermediate hyperhomocysteinemia

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ARTICLE INFO

Article history:
Received 31 December 2014
Accepted 31 December 2014
Available online 13 January 2015

Keywords:
Intermediate hyperhomocysteinemia
Dyrk1a gene transfer
Mice
Alanine aminotransferase
Apolipoproteins
Lecithin:cholesterol acyltransferase

ABSTRACT

Hyperhomocysteinemia results from hepatic metabolism dysfunction and is characterized by a high plasma homocysteine level, which is also an independent risk factor for cardiovascular disease. Elevated levels of homocysteine in plasma lead to hepatic lesions and abnormal lipid metabolism. Therefore, lowering homocysteine levels might offer therapeutic benefits. Recently, we were able to lower plasma homocysteine levels in mice with moderate hyperhomocysteinemia using an adenoviral construct designed to restrict the expression of DYRK1A, a serine/threonine kinase involved in methionine metabolism (and therefore homocysteine production), to hepatocytes. Here, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific *Dyrk1a* gene transfer on intermediate hyperhomocysteinemia and its associated hepatic toxicity and liver dysfunction. Commensurate with decreased plasma homocysteine and alanine aminotransferase levels, targeted hepatic expression of DYRK1A in mice with intermediate hyperhomocysteinemia resulted in elevated plasma paraoxonase-1 and lecithin:cholesterol acyltransferase activities and apolipoprotein A–I levels. It also rescued hepatic apolipoprotein E, J, and D levels. Further, Akt/GSK3/cyclin D1 signaling pathways in the liver of treated mice were altered, which may help prevent homocysteine-induced cell cycle dysfunction. *DYRK1A* gene therapy could be useful in the treatment of hyperhomocysteinemia in populations, such as end-stage renal disease patients, who are unresponsive to B-complex vitamin therapy.

1. Introduction

Cystathionine beta synthase (CBS) deficiency is the most common inborn error of one-carbon metabolism and is the cause of classical homocystinuria, a condition characterized by high levels of plasma homocysteine (hcy) or severe hyperhomocysteinemia (hhcy) [1]. Elevated plasma hcy, or hhcy, is categorized by range as moderate (15 to 30 μM), intermediate (30 to 100 μM), and severe (above 100 μM). Hyperhomocysteinemia is associated with increased risk for congenital disorders, including neural tube closure defects, heart defects, cleft lip/palate, Down syndrome, and multi-system abnormalities in adults [2].

hcy is a thiol-containing amino acid produced during metabolism of methionine (an essential amino acid supplied by dietary proteins) via the adenosylated compounds S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). The metabolism of hcy occurs at the junction of two pathways: remethylation, the process leading to the reconstruction of the methionine particle, and the transsulfuration pathway. Conversion of hcy to cystathionine is catalyzed by CBS, which is vitamin B6-dependent and functions first in the transsulfuration pathway. hcy can also revert to SAH via reversal of the S-adenosylhomocysteine hydrolase (SAHH) reaction [3]. Increased hcy synthesis and its slower intracellular utilization cause increased efflux into the blood. Hence, plasma hcy level is an important reflection of hepatic methionine metabolism and

Abbreviations: ALT, alanine aminotransferase; APO, apolipoprotein; CBS, cystathionine beta synthase; DCPIP, 2,6-dichlorophenolindophenol; hcy, homocysteine; HDLs, high-density lipoproteins; hhcy, hyperhomocysteinemia; HPLC, high-performance liquid chromatography; KYNA, kynurenic acid; LCAT, lecithin:cholesterol acyltransferase; NQO1, NAD(P)H: quinone oxidoreductase; PON-1, paraoxonase-1; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; VLDL, very low-density lipoprotein.

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http://dx.doi.org/10.1016/j.ymgmr.2014.12.007

of the rate of processes modified by B vitamins as well as activities of different enzymes [4].

Current treatment for CBS deficiency involves lowering hcy levels with a combination of vitamins, protein restriction, and cysteine supplementation [5–7]. Although treatments can be effective, the challenging dietary regimens often create problems with compliance. However, approximately 50% of patients with CBS deficiency are biochemically responsive to pyridoxine (vitamin B6) and show improvement in plasma hcy levels [8]. Therefore, drugs that could stimulate residual CBS enzyme activity could be useful in treating the disease.

One possible approach to stimulating CBS activity is by using gene therapy to deliver a gene (and, therefore, protein) that could increase the activity of existing CBS protein in the liver. We recently used an adenoviral construct designed to restrict overexpression of DYRK1A, a serine/threonine kinase involved in several steps of methionine metabolism, to hepatocytes of hhcy mouse models [9,12]. Indeed, we have demonstrated the involvement of hepatic Dyrk1a levels on hepatic SAHH and CBS activities by a mechanism dependent of NAD(P)H: quinone oxidoreductase (NQO1) activity and pyridoxal phosphate respectively [10–12]. Injection of the specific adenoviral *Dyrk1a* gene transfer construct to mice with moderate hhcy produced decreased plasma hcy levels, in agreement with restoration of key enzymes of hcy metabolism, SAHH and CBS. Pyridoxal phosphate, the metabolically active form of vitamin B6 and cofactor of CBS, was elevated, consistent with the increase in CBS activity [11].

As the central organ of metabolism, many metabolic diseases originate in the liver; however, clinical manifestations can be extrahepatic. In the case of hhcy, the liver plays not only a central role in the metabolism of methionine and contributes the high levels of plasma hcy, but also produces and degrades lipoproteins. High-density lipoproteins (HDLs) exert potent protective effects, including the prevention and correction of endothelial dysfunction by their anti-oxidative and their anti-inflammatory properties [13]. Commensurate with decreased plasma hcy levels, targeted hepatic expression of *Dyrk1a* by adenoviral gene transfer resulted in elevated activity of plasma paraoxonase-1 (PON-1), an HDL-associated protein that inactivates lipids in oxidized low-density lipoproteins, and increased plasma levels of apolipoprotein A–I (APO A–I), the major protein component of HDLs. Additionally, the Akt/GSK3 signaling pathways were rescued in the aorta of targeted mice, thus preventing hcy-induced endothelial dysfunction [11]. In the current study, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific *Dyrk1a* gene transfer on intermediate hhcy and on the associated hepatic toxicity by measuring plasma alanine aminotransferase (ALT) levels, and liver dysfunction by measuring lipid markers.

2. Materials and methods

2.1. Experimental animals

All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handling. Mice were maintained in a controlled environment with unlimited access to food and water on a 12 h light/dark cycle. Mice were fed a standard laboratory diet (CRM, Special Diets Services, Dietex, France Usine). This diet has a protein content of 19%, a methionine content of 2.700 mg kg^{-1}, a folic acid content of 4.41 mg kg^{-1}, and a vitamin B12 content of 0.082 mg kg^{-1}. The number of mice and suffering were minimized as possible. Mice heterozygous for targeted disruption of the *Cbs* gene ($Cbs^{+/-}$) were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) [12]. $Cbs^{+/-}$ mice, on a C57BL/6 background, were obtained by mating male $Cbs^{+/-}$ mice with female wild-type C57BL/6 ($Cbs^{+/+}$) mice. DNA isolated from tail biopsies of 4-week-old mice was subjected to genotyping of the targeted *Cbs* allele using polymerase chain reaction (PCR) [14]. The E1E3E4-deleted adenoviral vector "AdDYRK1A" was constructed to induce hepatocyte specific overexpression of DYRK1A as described previously [11]. Generation of the E1E3E4-deleted adenoviral vector "AdDYRK1A" and large-scale vector production were performed as described previously [15]. Before the experiments and to induce intermediate hhcy, female $Cbs^{+/-}$ mice, 3 at 4 months of age, were maintained for three months on the standard diet supplemented with 0.5% L-methionine (Sigma-Aldrich, France) in drinking water. Mice were divided into two groups for the last month, with one group receiving injection in the retro-orbital sinus with an adenoviral vector AdDYRK1A to have 2×10^{12} adenoviral particles/kg body weight and the second group receiving an equivalent dose of saline buffer [11]. Control mice, healthy control $Cbs^{+/+}$ mice also received an equivalent dose of saline buffer and were used as references to monitor hyperhomocysteinemic development. Five to twelve mice per experiment were used.

2.2. Preparation of serum samples, tissue collection, and plasma assays

Upon euthanization of mice by Ketamine/Xylazine intraperitoneal injection, blood samples were obtained by retro-orbital sinus sampling with heparinized capillaries, collected into tubes containing a 1/10 volume of 3.8% sodium citrate, and immediately placed on ice. Plasma was isolated by centrifugation at 2500 ×*g* for 15 min at 4 °C. Livers were harvested, snap-frozen, and stored at −80 °C until use. Plasma total hcy, defined as the total concentration of hcy after quantitative reductive cleavage of all disulfide bonds, was assayed using the fluorimetric high-performance liquid chromatography (HPLC) method as previously described [16]. Samples were treated with perchloric acid and kynurenic acid (KYNA) was quantified by LC-MS/MS with CTC-PAL autosampler on an Agilent 1200 series system with a quaternary pump, and MS detection was performed on an API 3200 MS/MS spectrometer (ABSciex) operated with a Turbo Ion Spray source [17]. KYNA used as standard was purchased from Sigma-Aldrich. Data were acquired and processed using Analyst software (V. 1.4.2). Plasma APO A–I protein levels were measured by ELISA (E90519M, Uscn, Life Science Inc.) according to the manufacturer's instructions. After the development of the colorimetric reaction, optical density (OD) at 450 nm was quantified by a microplate reader (Flex Station3, Molecular Device), and OD readings were converted to concentrations (μg/mL) on the basis of the standard curve obtained with APO A–I standard preparation. ALT was assayed using the Alanine Aminotransferase Activity Assay Kit (Sigma-Aldrich, France), based on the pyruvate generated.

2.3. Protein extraction and slot blot analysis

Liver protein extracts were prepared by homogenizing tissue in phosphate-buffered saline (PBS) with a cocktail of protease inhibitors (1 mM Pefabloc SC, 5 μg/mL E64, and 2.5 μg/mL Leupeptin). Homogenates were centrifuged at 12,500 ×*g* for 15 min at 4 °C. Supernatants were then assayed for protein concentrations with the Coomassie (Bradford) Protein Assay reagent (Bio-Rad). Since specificity of each primary antibody used was previously validated by Western blotting, protein extracts (40 μg) under reducing conditions were subjected to slot blotting on nitrocellulose transfer membrane PROTRAN® (Whatman). The membrane was then blocked in 10% nonfat dry milk in Tris–saline buffer (1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) and probed overnight at 4 °C with one of the following antibodies: anti-APO D (1/1000; Santa Cruz Biotechnology, Tebu, France), anti-APO E (1/1000; Santa Cruz Biotechnology), anti-APO J (1/1000; Santa Cruz Biotechnology), anti-DYRK1A (1/250; Abnova Corporation, Tebu, France), anti-Akt (1/1000; Santa Cruz Biotechnology), anti-phospho-Akt1/2/3 (Ser 473; 1/1000; Santa Cruz Biotechnology), anti-GSK (1/2000, Santa Cruz Biotechnology,), anti-phospho-GSK3 (ser21-9; 1/400, Cell Signaling, Ozyme, France), or anti-cyclin D1 (1/250, Cell Signaling). Horseradish peroxidase-conjugated secondary antibody and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) were used to detect specific proteins. Digitized images of the immunoblots obtained using an LAS-