Molecular Genetics and Metabolism xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

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

Oral aversion to dietary sugar, ethanol and glycerol correlates with alterations in specific hepatic metabolites in a mouse model of human citrin deficiency

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ARTICLE INFO

Article history:
Received 24 December 2016
Received in revised form 5 February 2017
Accepted 5 February 2017
Available online xxxx

Key words:
Adult-onset type II citrullinemia (CTLN2)
ATP
Dietary aversion
Citrin deficiency
Glycerol 3-phosphate
Cytosolic NADH

ABSTRACT

Mice carrying simultaneous homozygous mutations in the genes encoding citrin, the mitochondrial aspartate-glutamate carrier 2 (AGC2) protein, and mitochondrial glycerol-3-phosphate dehydrogenase (mGPD), are a phenotypically representative model of human citrin (a.k.a., AGC2) deficiency. In this study, we investigated the voluntary oral intake and preference for sucrose, glycerol or ethanol solutions by wild-type, citrin (Ctrn)-knockout (KO), mGPD-KO, and Ctrn/mGPD double-KO mice; all substances that are known or suspected precipitating factors in the pathogenesis of human citrin deficiency. The double-KO mice showed clear suppressed intake of sucrose, consuming less with progressively higher concentrations compared to the other mice. Similar observations were made when glycerol or ethanol were given. The preference of Ctrn-KO and mGPD-KO mice varied with the different treatments; essentially no differences were observed for sucrose, while an intermediate intake or similar to that of the double-KO mice was observed for glycerol and ethanol. We next examined the hepatic glycerol 3-phosphate, citrate, citrulline, lysine, glutamate and adenine nucleotide levels following forced enteral administration of these solutions. A strong correlation between the simultaneous increased hepatic glycerol 3-phosphate and decreased ATP or total adenine nucleotide content and observed aversion of the mice during evaluation of their voluntary preferences was found. Overall, our results suggest that the aversion observed in the double-KO mice to these solutions is initiated and/or mediated by hepatic metabolic perturbations, resulting in a behavioral response to increased hepatic cytosolic NADH and a decreased cellular adenine nucleotide pool. These findings may underlie the dietary predilections observed in human citrin deficient patients.

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Abbreviations: AGC, aspartate-glutamate carrier; AN, adenine nucleotide; ANOVA, analysis of variance; Asp, aspartate; bw, body weight; cGPD, cytosolic glycerol-3-phosphate dehydrogenase; CTLN2, adult-onset type II citrullinemia; Ctrn-KO, citrin (a.k.a., *Slc25a13*) knockout; FGF21, fibroblast growth factor 21; Glu, glutamate; GAPD, glyceraldehyde 3-phosphate dehydrogenase; G3P, glycerol 3-phosphate; GP, glycerophosphate; KO, knockout; Lac, lactate; LC/MS, liquid chromatography/mass spectrometry; Lys, lysine; MA, malate-aspartate; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; NS, not statistically significant; TCA, tricarboxylic acid; UPLC, ultra-performance liquid chromatography; wt, wild-type.

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[1] Posthumous.

http://dx.doi.org/10.1016/j.ymgme.2017.02.004
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Please cite this article as: T. Saheki, et al., Oral aversion to dietary sugar, ethanol and glycerol correlates with alterations in specific hepatic metabolites in a mouse model ..., Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.02.004

1. Introduction

Human citrin deficiency, also known as mitochondrial aspartate-glutamate carrier 2 (AGC2) deficiency, is one of the most well-characterized genetic disorders associated with a member of the large SLC25A family of mitochondrial transporter proteins [1,2]. Now an established disease entity [3], citrin deficiency is caused by mutations in *SLC25A13* that result in at least two previously distinct clinical presentations: adult-onset type II citrullinemia (CTLN2) characterized by hyperammonemia [4], and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) that leads to multiple aminoacidemias, galactosemia, hypoproteinemia and jaundice [5–7]. Citrin deficiency may also lead to additional consequences throughout life including growth retardation and hypoglycemia in infancy, fatty liver, hypertriglyceridemia, pancreatitis, and hepatocellular carcinoma [8–18], while a third phenotype, abbreviated FTTDCD and defined as failure to thrive and dyslipidemia [19,20] in an otherwise healthy individual, has also been described. The various reported phenotypes of citrin deficiency are not mutually exclusive and represent overlapping but distinct clinical features that emerge at different ages throughout the lives of patients (or in the case of CTLN2, only in a subset of patients).

Citrin, or AGC2, has been shown to be the liver-type isoform of the mitochondrial aspartate (Asp)-glutamate (Glu) carrier (AGC) [21] that participates in the synthesis of protein, nucleotides and urea within the cytosol through supplying mitochondrial Asp, while another isoform, aralar or AGC1, is the brain/skeletal muscle-type isoform. In addition, both AGCs play an important role in aerobic glycolysis as a member of the malate-Asp (MA) shuttle that, together with the glycerophosphate (GP) shuttle, transports cytosolic NADH equivalents into the mitochondria to synthesize ATP via oxidative phosphorylation, and, in the case of citrin, in gluconeogenesis from lactate (Lac; due to its stoichiometric relationship with NADH [22]).

Our initial studies of the homologous-recombination-generated *Slc25a13*-knockout (KO), or Ctrn-KO, mouse demonstrated metabolic perturbations in many of the pathways in which citrin was predicted to play a role, through in vitro assays and liver perfusion experiments [23]. Despite these findings, however, the mice failed to exhibit an observable phenotype relevant to human citrin deficiency. Based on published reports that rodent liver contains much higher GP shuttle activity compared to human liver [24–26], we have established a more suitable mouse model of human citrin deficiency [27] through breeding Ctrn-KO and Gpd2-KO (a.k.a., mitochondrial glycerol 3-phosphate dehydrogenase or mGPD) mice, creating Ctrn/mGPD double-KO mice. The double-KO mouse shows sustained elevations in plasma citrulline, and hyperammonemia under fed conditions, as well as hypoglycemia and fatty liver under fasted conditions; all mimicking symptoms reminiscent of human citrin deficiency.

The most characteristic features of the double-KO mouse are exasperation of the hyperammonemia following oral sucrose administration [27], and reduced consumption and body weight when fed a synthetic diet (AIN-93M) containing 72% carbohydrate content (i.e., approximately 20% more carbohydrates than standard laboratory chow diet CE2) that could be reversed by supplementing casein to increase the protein content [28]. These finding are in keeping with the reported dietary predilections of CTLN2 patients for foods high in protein and fat (such as beans and nuts), and avoidance of foods high in carbohydrates (such as cooked rice and sweets). Formal nutritional assessment of apparently healthy, citrin-deficient subjects have substantiated these findings; patients show a marked decrease in carbohydrate intake that accounts for a smaller proportion of carbohydrates contributing to their total energy intake (PFC ratio) [29]. Other published studies of CTLN2 patients have reported similar findings [30], and the clinical relevance of these observations are emphasized by a report of a CTLN2 patient that clearly showed further encephalopathic deterioration after initiation of a conventional low-protein diet therapy for chronic liver failure [31]. Furthermore, in addition to patients describing feeling ill after eating carbohydrate-rich foods such as rice and sweets, and experiencing symptoms of nausea, gastrointestinal discomfort and drowsiness, several citrin deficiency patients have also been reported to suffer disturbances of consciousness or abnormal behaviors following ingestion of alcohol [32,33], or have had worsening symptoms resulting in death shortly after the administration of glycerol and fructose [34]. Collectively, these findings point to the importance of dietary and other exogenous triggers in the pathogenesis of citrin deficiency.

To investigate the adverse reactions of citrin-deficient patients to carbohydrates, glycerol and ethanol, all substrates known to generate hepatocyte cytosolic NADH, we have examined the voluntary intake and preference of wild-type (wt), Ctrn-KO, mGPD-KO and Ctrn/mGPD double-KO mice for solutions of sucrose, glycerol, or ethanol versus water by a voluntary two-bottle test system. The double-KO mice show a clear suppression of intake and aversion to sucrose, glycerol and ethanol compared to wt mice, with the Ctrn-KO and mGPD-KO mice showing intakes and preferences similar to wt mice for sucrose, but intermediate or similar to the double-KO mice for glycerol and ethanol.

To determine whether the suppressed intake of the solutions correlated with specific hepatic metabolite changes in the mice, we examined the adenine nucleotide (AN) content in addition to other metabolites that have previously been identified as being altered following enteral sucrose administration [35]. From our analyses, the suppressed intake of the solutions occur under similar conditions to when enteral administration resulted in consistently increased hepatic glycerol 3-phosphate (G3P) and decreased ATP or total AN pools, suggesting that the highly reduced state of cytosolic NAD together with a decreased hepatocyte ATP or total AN pools may underlie the dietary aversion observed in the Ctrn/mGPD double-KO mice and human citrin deficient patients.

2. Materials and methods

2.1. Animals

All wt, Ctrn-KO, mGPD-KO and Ctrn/mGPD double-KO mice used were congenic on the C57BL/6J background. Mice were generated using the breeding scheme described previously by Saheki et al. [27]. Briefly, mGPD-KO and double-KO mice were obtained by mating heterozygous Ctrn-KO/homozygous mGPD-KO ($Ctrn^{+/-}$/$mGPD^{-/-}$) mice, while wt and Ctrn-KO mice were generated by mating heterozygous Ctrn-KO ($Ctrn^{+/-}$/$mGPDH^{+/+}$) mice. Genotype analysis was performed by duplex PCR with DNA extracted from ear punches using procedures specific for each of the targeted mutations in the Ctrn-KO [23] and mGPD-KO [36] mice, respectively. For the detection of the endogenous Ctrn (*Slc25a13*) and gene-targeted alleles, primers slc-B (5′-CCAAACTACCTTTGCAGACTT-3″), slc-F (5′-TGGGTTCTA TTGCTGGAGGT-3′) and Neo-F (5′-TACCGGTGGATGTGGAATGT-3′) were used. For detection of the mGPD (*Gpd-2*) and gene-targeted alleles, primers 2–15 (5″-TCGCTCCTCATTTATCAGCTCCG-3″), 2–16 (5″-ACAGGTGTTCACCTACACGCTCC-3″) and C2 (5″-TGGGAATGTGTGCGAGGCCAGAGGCCAC-3″) were used.

2.2. Animal care and treatment

All mice were maintained at a constant temperature (23 ± 1 °C) on a 12-h light/dark cycle (light on, 8:00 am to 8:00 pm) with free access to water and CE2 chow (24.9% protein, 4.6% fat, and 51% nitrogen-free extracts providing 343 kcal/100 g; CLEA Japan, Tokyo, Japan). Mice used for the experiments were analyzed between 80 and 160 days of age. Within a given experiment, both male and female mice were assessed separately, and then data pooled together (approximate equal ratio) when no statistical differences were found for the parameters measured.

In the experiments to assess the animals' oral voluntary intake and preference for different test solutions, solutions of sucrose (1–20% *w*/

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