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Experimental evidence that overexpression of NR2B glutamate receptor subunit is associated with brain vacuolation in adult glutaryl-CoA dehydrogenase deficient mice: A potential role for glutamatergic-induced excitotoxicity in GA I neuropathology

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ABSTRACT

Glutaric aciduria type I (GA I) is biochemically characterized by accumulation of glutaric and 3-hydroxyglutaric acids in body fluids and tissues, particularly in the brain. Affected patients show progressive cortical leukoencephalopathy and chronic degeneration of the basal ganglia whose pathogenesis is still unclear. In the present work we investigated parameters of bioenergetics and redox homeostasis in various cerebral structures (cerebral cortex, striatum and hippocampus) and heart of adult wild type ($Gcdh^{+/+}$) and glutaryl-CoA dehydrogenase deficient knockout ($Gcdh^{-/-}$) mice fed a baseline chow. Oxidative stress parameters were also measured after acute lysine overload. Finally, mRNA expression of NMDA subunits and GLT1 transporter was determined in cerebral cortex and striatum of these animals fed a baseline or high lysine (4.7%) chow. No significant alterations of bioenergetics or redox status were observed in these mice. In contrast, mRNA expression of the NR2B glutamate receptor subunit and of the GLT1 glutamate transporter was higher in cerebral cortex of $Gcdh^{-/-}$ mice. Furthermore, NR2B expression was markedly elevated in striatum of $Gcdh^{-/-}$ animals receiving chronic Lys overload. These data indicate higher susceptibility of $Gcdh^{-/-}$ mice to excitotoxic damage, implying that this pathomechanism may contribute to the cortical and striatum alterations observed in GA I patients.

1. Introduction

Glutaric aciduria type I (GA I, McKusick 23167, OMIM # 231670) is a cerebral organic aciduria caused by inherited deficiency of the activity of mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7). Affected patients accumulate glutaric (GA) and 3-hydroxyglutaric (3-HGA) acids in the brain and body fluids [1–4]. Clinically, they have predominantly neurological symptoms, such as dystonia, spasticity, loss of motor control, seizures and coma. In untreated patients, massive bilateral striatum degeneration often occurs after encephalopathic crises triggered by catabolic states within the first four years of life [4]. Moreover, although older patients appear to be less prone to acute episodes, they may develop progressive neurologic dysfunction [5–7]. Neuroradiological findings include frontotemporal atrophy associated with macrocephaly at birth, as well as progressive cortical frontotemporal hypoplasia with white matter abnormalities (leukoencephalopathy), chronic subdural effusions, hematomas, as well as acute striatal degeneration with loss of medium spiny neurons and astrogliosis [1,6,8]. Chronic treatment with low lysine (Lys) intake, carnitine supplementation, and emergency treatment during catabolic states have significantly improved the clinical outcome of these patients, suggesting that accumulation of Lys derivatives (GA and 3-HGA) contributes to a severe outcome in these patients [9].

Although the exact mechanisms of brain damage in GA I are so far poorly known, many in vitro and in vivo experiments implicate GA and 3-HGA as neurotoxins in this disease, causing excitotoxicity, oxidative damage and impairment of cellular energy metabolism [4,10–31]. Many different neurological models have been used in these investigations, and one promising model is the knockout mouse, which, like human patients, appears to be more vulnerable to an acute episode at earlier age. $Gcdh^{-/-}$ mice accumulate high levels of GA and 3-HGA but do not show spontaneous striatal neurodegeneration or relevant neurological symptoms unless they are fed a high Lys chow [4,6,32,33]. Thus, it seems that the neurological damage in GA I is dependent on GA and/or 3-HGA-mediated neurotoxicity [26]. The neuropathological findings in

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these animals fed high Lys chow were comparable to those of human patients, including neuronal loss and vacuolation, blood–brain barrier breakdown, myelin disruption and gliosis, mostly in the striatum and deep cortex [34,35].

Various studies performed in infant and adolescent *Gcdh*$^{-/-}$ mice (up to thirty days of life) revealed disruption of the redox state and alterations of the glutamatergic system, with mild bioenergetics disturbance in the brain of these animals [36–43]. Here we extend these studies to adult *Gcdh*$^{-/-}$ mice. We have measured many parameters of redox homeostasis and energy metabolism in various tissues of adults and demonstrated only modest differences in the knockout mice. Finally, we evaluated mRNA expression of various NMDA glutamate receptor subunits and of the GLT1 glutamate transporter, and saw differences in the knockout animals that suggest progressive encephalopathy in adult mice, and perhaps people, may be due to excitotoxic effects of increased GA and 3-HGA.

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffer for each technique.

2.2. Animals

Gcdh$^{+/+}$ and *Gcdh*$^{-/-}$ littermates, both of C129SvEv background, were generated from heterozygotes and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1 °C), with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). Ninety-day-old male and female *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice, corresponding to adult humans, were used in all experiments.

2.3. Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85–23, revised in 2011, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

2.1. Chronic and acute Lys overload and outcome of *Gcdh*$^{-/-}$ mice

For the experiments designed to measure mRNA expression of NMDA receptor subunits and the GLT1 transporter, forty-five-day-old *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice received a baseline chow (containing 20% protein and 0.9% Lys) or a high Lys chow (containing 20% protein and 4.7% Lys) for 45 days. An acute intraperitoneal injection of Lys (8 μmol/g body weight) was also carried out in 90-day-old *Gcdh*$^{-/-}$ mice and some oxidative stress parameters were measured in the cerebral cortex 24 h after injection. All *Gcdh*$^{-/-}$ animals were asymptomatic throughout these treatments and exhibited no mortality.

2.2. Tissue preparation for oxidative stress and bioenergetics measurement

Mice were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and transcardiacally perfused for 5 min with saline solution. After perfusion, the brain and heart were rapidly removed, and striatum, cerebral cortex and hippocampus were dissected in a Petri dish placed on ice. These tissues were homogenized in 9 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl for the oxidative stress parameters, and in 19 volumes (1:20, w/v) of SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI · mL^{-1} heparin) for the bioenergetics measurements. Homogenates were centrifuged at 750 ×*g* for 10 min at 4 °C to precipitate nuclei and cell debris [44]. The pellet was discarded and the supernatant, which included mitochondria, was retained for the measurements.

2.3. Oxidative stress parameters

2.3.1. Malondialdehyde (MDA) levels

MDA levels were measured according to the method described by Yagi [45] with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% thiobarbituric acid (TBA) in 7.1% sodium sulfate were added to 100 μL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read using 515 nm and 553 nm as excitation and emission wavelengths, respectively. A calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein.

2.3.2. 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [46] by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl and incubated with tissue slices (30 mg) for 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of reactive species. The DCF fluorescence intensity parallels the amount of reactive species present. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was prepared with DCF standards (0.25–10 mM) and the levels of reactive species were calculated from the curve and expressed as pmol DCF formed/mg protein.

2.3.3. Sulfhydryl content

This assay is based on the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [47]. Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of tissue supernatants containing 0.3 mg of protein. This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were calculated using TNB molar extinction coefficient and expressed as nmol/mg protein.

2.3.4. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [48]. One volume of metaphosphoric acid was added to 150 μL of tissue supernatant, which was centrifuged for 10 min at 7000 ×*g*. Then 30 μL of the resulting supernatant was diluted with 70 μL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. This preparation was incubated with o-phthaldialdehyde (1 mg/mL in methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Concentrations were calculated from a calibration curve of a GSH standard (0.001–1 mM) and expressed as nmol/mg protein.