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Induction of oxidative stress in brain of glutaryl-CoA dehydrogenase deficient mice by acute lysine administration

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ABSTRACT

In the present work we evaluated a variety of indicators of oxidative stress in distinct brain regions (striatum, cerebral cortex and hippocampus), the liver, and heart of 30-day-old glutaryl-CoA dehydrogenase deficient (*Gcdh*$^{-/-}$) mice. The parameters evaluated included thiobarbituric acid-reactive substances (TBA-RS), 2-7-dihydrodichlorofluorescein (DCFH) oxidation, sulfhydryl content, and reduced glutathione (GSH) concentrations. We also measured the activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PD). Under basal conditions glutaric (GA) and 3-OH-glutaric (3OHGA) acids were elevated in all tissues of the *Gcdh*$^{-/-}$ mice, but were essentially absent in WT animals. In contrast there were no differences between WT and *Gcdh*$^{-/-}$ mice in any of the indicators or oxidative stress under basal conditions. Following a single intra-peritoneal (IP) injection of lysine (Lys) there was a moderate increase of brain GA concentration in *Gcdh*$^{-/-}$ mice, but no change in WT. Lys injection had no effect on brain 3OHGA in either WT or *Gcdh*$^{-/-}$ mice. The levels of GA and 3OHGA were approximately 40% higher in striatum compared to cerebral cortex in Lys-treated mice. In the striatum, Lys administration provoked a marked increase of lipid peroxidation, DCFH oxidation, SOD and GR activities, as well as significant reductions of GSH levels and GPx activity, with no alteration of sulfhydryl content, CAT and G6PD activities. There was also evidence of increased lipid peroxidation and SOD activity in the cerebral cortex, along with a decrease of GSH levels, but to a lesser extent than in the striatum. In the hippocampus only mild increases of SOD activity and DCFH oxidation were observed. In contrast, Lys injection had no effect on any of the parameters of oxidative stress in the liver or heart of *Gcdh*$^{-/-}$ or WT animals. These results indicate that in *Gcdh*$^{-/-}$ mice cerebral tissue, particularly the striatum, is at greater risk for oxidative stress than peripheral tissues following Lys administration.

1. Introduction

Glutaric aciduria type I (GA I, OMIM # 231670) is a cerebral organic aciduria caused by severe deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) activity that catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA [1]. GCDH deficiency results in tissue accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA), and the derivative glutarylcarnitine. Lysine (Lys) oxidation seems to be quantitatively the most important pathway for the formation of these metabolites [2–4]. At birth, affected individuals present with macrocephaly associated with frontotemporal hypoplasia. Most untreated patients develop acute encephalopathic crises that lead to permanent striatal destruction and dystonia. The vast majority of these crises occur between 6 months and 3 years of age, and are frequently

Abbreviations: CAT, catalase; DCF-DA, 2-7-dihydrodichlorofluorescein diacetate; DCFH, 2-7-dihydrodichlorofluorescein; DTNB, 5-5-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GA I, glutaric aciduria type I; GA, glutaric acid; *Gcdh*$^{-/-}$, deficient knockout mice; GCDH, glutaryl-CoA dehydrogenase; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose-6-phosphate dehydrogenase; 3OHGA, 3-hydroxyglutaric acid; IP, intra-peritoneal; KO, knockout; Lys, lysine; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NADP^{+}, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; RS, reactive species; SPSS, Statistical Package for the Social Sciences; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid-reactive substances; WT, wild type.

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precipitated by catabolic states, such as infections, fever and fasting. However, a number of patients who never have an acute crisis nonetheless develop dystonia due to striatal injury [5–7]. Another characteristic feature of GA I is delayed maturation of cerebral white matter, which also occurs in patients who never have an acute encephalopathic crisis. Pathologically the acute striatal degeneration in GA I is associated with loss of medium spiny neurons. Extra-striatal neuropathology commonly seen in GA I includes subdural and retinal hematomas, and a spongiform myelinopathy which seems to progresses with age [1,6,8–10].

Although the exact pathomechanisms underlying the brain damage of GA I are not fully understood, a great body of evidence indicates that GA and 3OHGA are involved in its pathogenesis, causing excitotoxicity, oxidative stress and mitochondrial dysfunction [4,11–33]. It is emphasized that most evidence revealing these pathomechanisms was based on in vitro experiments performed in fresh cerebral cortex and striatum, or in neuronal and astrocytic cell cultures from rats and chick embryos with normal GCDH activity, which makes the pathophysiological relevance of these works uncertain.

Recently a knockout (KO) model of GA I was developed in mice by replacing the *Gcdh* gene with an in-frame beta-galactosidase cassette [31]. Exposing these animals to high protein or Lys intake resulted in elevated serum and brain GA accumulation, as well as neuronal loss, myelin disruption and gliosis mostly in the striatum and deep cortex [33,34]. A chronic increase in oral Lys intake to weanling (4-week-old) *Gcdh*$^{-/-}$ mice provoked an increase of brain Lys and GA levels after 48 h of Lys exposure. Disrupted mitochondrial function, evidenced by mitochondrial swelling, accumulation of acetyl-coenzyme A, decrease of ATP, phosphocreatine and coenzyme A, as well as a reduction of alpha-ketoglutarate, glutamate, glutamine and GABA concentrations were also found in the cerebral cortex of the Lys-treated *Gcdh*$^{-/-}$ mice [34].

Considering that to the best of our knowledge practically nothing has been reported on cellular redox homeostasis in the *Gcdh*$^{-/-}$ genetic model, in the present study we evaluated a large spectrum of important parameters of oxidative stress in various brain regions (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) of WT and *Gcdh*$^{-/-}$ animals in order to clarify whether oxidative stress is involved in the pathogenesis, and more specifically in the brain damage of GA I. We measured thiobarbituric acid-reactive substances (TBA-RS), 2-7-dihydrodichlorofluorescein (DCFH) oxidation, sulfhydryl content, reduced glutathione (GSH) concentrations and the activities of the antioxidant enzymes glutathione peroxidise (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PD) in brain regions (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) of WT and *Gcdh*$^{-/-}$ mice while on standard mouse chow, and after acute lysine administration in order to clarify whether oxidative stress is involved in the pathogenesis and more specifically in the brain damage of GA I.

2. Materials 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 buffers for each technique.

2.2. Animals

Gcdh$^{-/-}$ and WT mice littermate controls, both of C129SvEv background, were generated from heterozygotes and maintained at Fundação Estadual de Produção e Pesquisa em Saúde (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) colony room, with free access to water and commercial chow containing 20% (w/w) protein, and 0.9% lysine (SUPRA, Porto Alegre, RS, Brazil). Thirty-day-old male WT and *Gcdh*$^{-/-}$ mice from F1 and F2 generations 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 1996, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Federal University of Rio Grande do Sul. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

2.4. Lys administration

A group of WT and *Gcdh*$^{-/-}$ animals were given one intraperitoneal (i.p.) injection of Lys (8 μmol/g) in order to investigate whether an acute Lys overload could induce oxidative stress in a model of GCDH deficiency. It is emphasized that catabolism leading to increased generation of Lys occurs during fasting/infections in GA I patients and may lead to striatal degeneration whose mechanisms are still poorly known. Lys, GA and 3OHGA concentrations were determined 1 h, 2 h and 24 h after Lys administration in striatum and cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Protein concentrations were also determined in brain homogenates from WT and *Gcdh*$^{-/-}$ animals. The obtained data revealed that these concentrations did not differ between controls and the GA I KO mice model and were approximately 3 mg/mL of protein. Lys concentrations were measured by cation-exchange chromatography on a Biochrom 30+ Amino Acid Analyzer, whereas GA and 3OHGA levels were quantified by stable isotope dilution GC/MS using an Agilent Technologies 6890N Gas Chromatograph equipped with a 5973N Mass Selective Detector. The internal standards were (2,2,4,4-D4) GA and (2,2,4,4-D4) 3OHGA. The parameters of oxidative stress were measured 24 h after Lys injection in order to study the medium to long-term effects of increased brain GA concentrations. It is important to emphasize that at this time GA concentrations returned to basal levels in the *Gcdh*$^{-/-}$ mice, so that our results could not be attributed to the presence of GA in the assays.

2.5. Tissue preparation

The mice were anesthetized with the mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardiacally perfused during 5 min with saline solution. After perfusion, brain, liver and heart were rapidly removed and placed on a Petri dish on ice. The olfactory bulb, pons, medulla, and cerebellum were discarded, and the cerebral cortex, striatum and hippocampus dissected and weighed. The tissues were homogenized in 9 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C to discard nuclei and cell debris [35]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters. Tissue slices (400 μm) were also prepared from the cerebral and peripheral structures for DCFH oxidation measurement.

2.6. Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS levels were measured according to the method described by Yagi [36] with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% 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