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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

was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein. Results were expressed as percentage of controls.

2.7. 2-7-Dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [37] 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) during 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 (RS). The DCF fluorescence intensity parallels to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.2510 mM) and the levels of RS were calculated as pmol DCF formed/mg protein. Results were expressed as percentage of controls.

2.8. Sulfhydryl content

This assay is based on the reduction of 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [38]. 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 and expressed as nmol/mg protein.

2.9. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [39]. Tissue supernatants with approximately 0.3 mg of protein were diluted (1:20, v/v) in 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL in methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein. Results were expressed as percentage of controls.

2.10. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [40] using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.11. Glutathione reductase (GR) activity

GR activity was measured according to Calberg and Mannervik [41] using oxidized glutathione (GSSG) and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid, 1 mM GSSG, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GR unit (U) is defined as 1 μmol of GSSG reduced per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.12. Catalase (CAT) activity

CAT activity was assayed according to Aebi [42] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.13. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [43] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on $O_2^{\bullet -}$, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and tissue supernatants (approximately 1 μg of protein). A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results were calculated as U/mg protein and expressed as percentage of controls.

2.14. Glucose-6-phosphate dehydrogenase (G6PD) activity

G6PD activity was measured by the method of Leong and Clark [44] in a reaction mixture (1 mL) containing 100 mM Tris–HCl pH 7.5, 10 mM MgCl2, 0.5 mM NADP + and tissue supernatants (approximately 3 μg of protein). The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PD unit corresponds to 1 mmol of substrate transformed per minute and the specific activity was calculated as units per mg protein and expressed as percentage of controls.

2.15. Protein determination

Protein levels were measured by the method of Lowry et al. [45] using bovine serum albumin as standard.

2.16. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples. Only significant *t* values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Effect of i.p. lysine injection on brain levels of lysine, glutaric acid and 3-hydroxyglutaric acid

In the cerebral cortex the levels of Lys were similar in WT and *Gcdh*$^{-/-}$ mice when maintained on standard lab chow, whereas GA and 3OHGA concentrations were undetectable in WT (results not

Table 1
Concentrations of lysine (Lys), glutaric acid (GA) and 3-hydroxyglutaric acid (3OHGA) in the cerebral cortex and striatum of WT and *Gcdh*$^{-/-}$ mice 2 h after a single intraperitoneal injection of lysine (8 μmol/g).

	Cerebral cortex			Striatum	
	WT plus Lys	*Gcdh*$^{-/-}$	*Gcdh*$^{-/-}$ plus Lys	WT plus Lys	*Gcdh*$^{-/-}$ plus Lys
Lys	48.5 ± 3.1	54 ± 1.8	85.2 ± 4.32** ##	63.5 ± 3.7	109 ± 5.76***
GA	248 ± 55.8	3709 ± 442	4974 ± 346*** ##	438 ± 63.4	6332 ± 355***
3OHGA	37 ± 6.72	340 ± 4.8	364 ± 98**	65.8 ± 23.2	491 ± 38.8***

Values are mean ± standard deviation for 4 independent experiments (animals). *P<0.05, **P<0.01, ***P<0.001, compared to WT plus Lys mice; ##P<0.01 compared to *Gcdh*$^{-/-}$ (Student's *t*-test for unpaired samples). We did not measure Lys, GA and 3OHGA in WT mice without Lys administration. Results are expressed as μmol/g brain.

shown). In contrast, cortical levels of GA and 3OHGA were strongly increased in the *Gcdh*$^{-/-}$ mice (15- and 9-fold, respectively), as has been previously observed in these animals [33,34]. Two hours after an acute i.p. injection of Lys the levels of Lys and GA in the cerebral cortex of *Gcdh*$^{-/-}$ animals were increased by an average 1.6 and 1.35 fold, respectively, with no significant change in the level of 3OHGA (Table 1). The levels of all three metabolites were similar at 1 and 2 h post Lys injection, and returned to baseline by 24 h (data not shown).

In the striatum, the levels of Lys, GA, and 3OHGA in *Gcdh*$^{-/-}$ mice were all significantly increased in comparison to WT mice 2 h after i.p. Lys injection. Striatal metabolite levels were also higher than those seen in the cerebral cortex of *Gcdh*$^{-/-}$ mice following i.p. Lys injection (Table 1). Data are not available for the cortical or striatal levels of these metabolites in WT mice that did not receive Lys supplementation and for the striatum of non-injected *Gcdh*$^{-/-}$ mice.

3.2. Effect of Lys injection on measures of oxidative stress

We first observed no differences between untreated WT and *Gcdh*$^{-/-}$ mice in any of the measured parameters at baseline (results not shown). We then evaluated the effect of a single i.p. injection of Lys on these parameters since this injection resulted in elevation of brain levels of GA and 3OHGA.

3.3. Lys administration induces brain lipid peroxidation in Gcdh$^{-/-}$ mice

In comparison to WT mice, the levels of malondialdehyde (TBA-RS) in *Gcdh*$^{-/-}$ mice were 50% higher in the cerebral cortex [$t_{(8)} = -3.952$; P<0.01] and 40% higher in the striatum [$t_{(12)} = -4.277$; P<0.001] following a single i.p. injection of Lys (Fig. 1). In contrast, TBA-RS levels

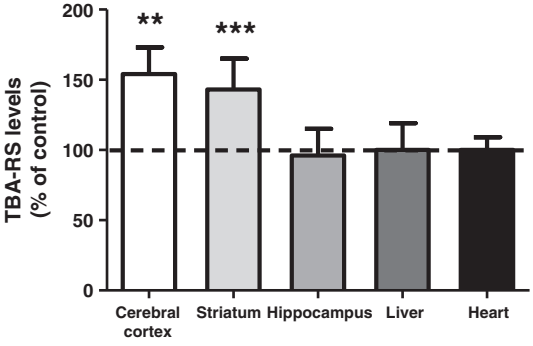

Fig. 1. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on thiobarbituric acid-reactive substances (TBA-RS) levels in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 0.74 ± 0.18; [nmol TBA-RS/mg protein]). **P<0.01, ***P<0.001, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

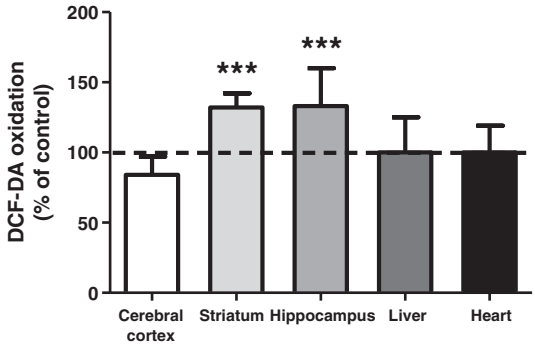

Fig. 2. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on 2-7-dihydrodichlorofluorescein (DCFH) oxidation in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 20.99 ± 2.34; [pmol DCF/mg protein]). ***P<0.001, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

in hippocampus, liver and heart from were the same in WT and *Gcdh*$^{-/-}$ mice 24 h after Lys injection.

3.4. Lys administration provokes brain DCFH oxidation in Gcdh$^{-/-}$ mice

Next, we assessed the influence of Lys injection on DCFH oxidation in cerebral cortex, striatum, hippocampus, liver and heart from *Gcdh*$^{-/-}$ mice. We observed that Lys significantly increased DCFH oxidation up to 30% in striatum [$t_{(12)} = -8.035$; P<0.001] and hippocampus [$t_{(9)} = -2.209$; P<0.001] of *Gcdh*$^{-/-}$ mice (Fig. 2). There were no differences between WT and *Gcdh*$^{-/-}$ mice in the amount of DCFH oxidation in cerebral cortex, liver and heart.

3.5. Lys administration does not alter sulfhydryl content in Gcdh$^{-/-}$ mice

We also investigated the effect of Lys administration on sulfhydryl content in striatum, cerebral cortex, hippocampus, liver and heart from *Gcdh*$^{-/-}$ and WT mice. It can be observed in Table 2 that Lys injection did not modify these parameters in all examined structures.

3.6. Lys administration reduces brain glutathione (GSH) concentrations in Gcdh$^{-/-}$ mice

We also investigated the effect of Lys administration on GSH concentrations in mice tissues. It can be observed in Fig. 3 that Lys injection significantly decreased (up to 15%) the concentrations of GSH in cerebral cortex [$t_{(10)} = 5.025$; P<0.001] and in striatum (up to 25%) [$t_{(8)} = 5.026$; P<0.001], with no alteration of this parameter in hippocampus, liver and heart.

Table 2
Sulfhydryl content of cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after intraperitoneal lysine injection (8 μmol/g).

	Sulfhydryl content	
	WT	*Gcdh*$^{-/-}$
Cerebral cortex	36.13 ± 6.92	36.76 ± 6.75
Striatum	59.54 ± 7.51	59.77 ± 12.61
Hippocampus	21.54 ± 9.39	24.52 ± 5.03
Liver	68.55 ± 4.98	72.89 ± 9.85
Heart	49.84 ± 6.19	50.28 ± 5.97

Values are mean ± standard deviation for 6 independent experiments (animals). Results are expressed as nmol/mg protein. No significant differences were found between groups (Student's *t*-test for unpaired samples).

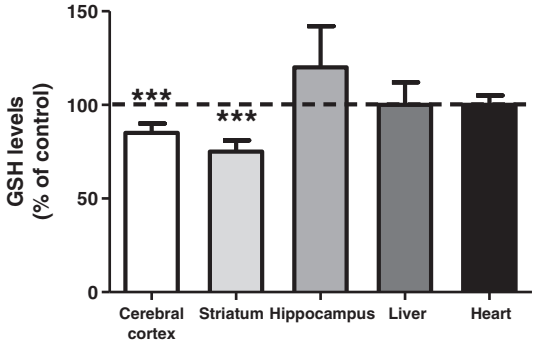

Fig. 3. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on reduced glutathione (GSH) levels in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 7.62 ± 0.45; [nmol GSH/mg protein]). ***$P<0.001$, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

3.7. Lys administration alters the activities of the antioxidant enzymes glutathione peroxidase, glutathione reductase and superoxide dismutase in brain of Gcdh$^{-/-}$ *mice*

We investigated the effect of Lys administration on the activities of GPx, GR, CAT, SOD and G6PD in cerebral cortex, striatum, hippocampus, liver and heart from *Gcdh*$^{-/-}$ mice. Our results demonstrate that a single Lys injection provoked an increase of SOD activity in cerebral cortex (28%) [$t_{(7)} = -3.192$; $P<0.05$], striatum (100%) [$t_{(12)} = -5.922$; $P<0.001$] and hippocampus (22%) [$t_{(8)} = 2.613$; $P<0.05$] (Fig. 4D), with no alteration in liver and heart (results not shown). Moreover, the activity of GPx was significantly decreased (41%) [$t_{(12)} = 6.947$; $P<0.001$] (Fig. 4A) and GR activity was increased (45%) only in the striatum after Lys administration ([$t_{(12)} = -4.708$; $P<0.001$] (Fig. 4B), with no alterations in cerebral cortex, hippocampus, liver and heart (results not shown). Finally, CAT (Fig. 4C) and G6PD (Fig. 4E) activities were not altered by Lys administration in any of the tissues examined.

4. Discussion

In the present work we searched for alterations of cellular redox homeostasis in central and peripheral tissues of 30-day-old *Gcdh*$^{-/-}$ mice. We first observed that the oxidative stress parameters did not differ between *Gcdh*$^{-/-}$ and WT mice receiving a normal diet (0.9% Lys). These data reflect that at this age under basal conditions there is no induction of oxidative stress in the *Gcdh*$^{-/-}$ mice compared to the WT mice.

We also investigated the effects of an acute Lys injection on oxidative damage, reactive oxygen species formation and the antioxidant defenses in the *Gcdh*$^{-/-}$ mice since temporary high Lys generation due to enhanced proteolysis occurs in GA I patients during episodes of metabolic decompensation, which are generally followed by acute striatum degeneration. Lys administration led to increased brain levels of GA soon after injection, with the highest concentrations achieved in the striatum from the *Gcdh*$^{-/-}$ mice. We also found that Lys administration resulted in increased reactive species production, lipid peroxidation and impaired antioxidant defenses following Lys supplementation in the brain of the *Gcdh*$^{-/-}$ animals. Furthermore, the observed effects were more pronounced in the striatum, but were also seen in the cerebral cortex and in hippocampus to a much lesser degree, with no alteration in liver and heart in, as compared to the WT mice.

Our observations that Lys overload provoked marked lipid peroxidation in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice was based on the increased TBA-RS levels that reflect a high formation of malondialdehyde, an end product of membrane fatty acid oxidation [46]. It is feasible that the lipid oxidative damage following Lys supplementation could be secondary to elevated amounts of reactive species since DCFH-DA oxidation, a measurement of these species, was found increased especially in the in the striatum of the Lys-injected *Gcdh*$^{-/-}$ animals.

Regarding to the antioxidant defenses, the concentrations of GSH, the major brain antioxidant with both cytoplasmatic and mitochondrial GSH pools [47], were reduced in the striatum and cerebral cortex of the *Gcdh*$^{-/-}$ animals that received Lys. Considering that GSH is an

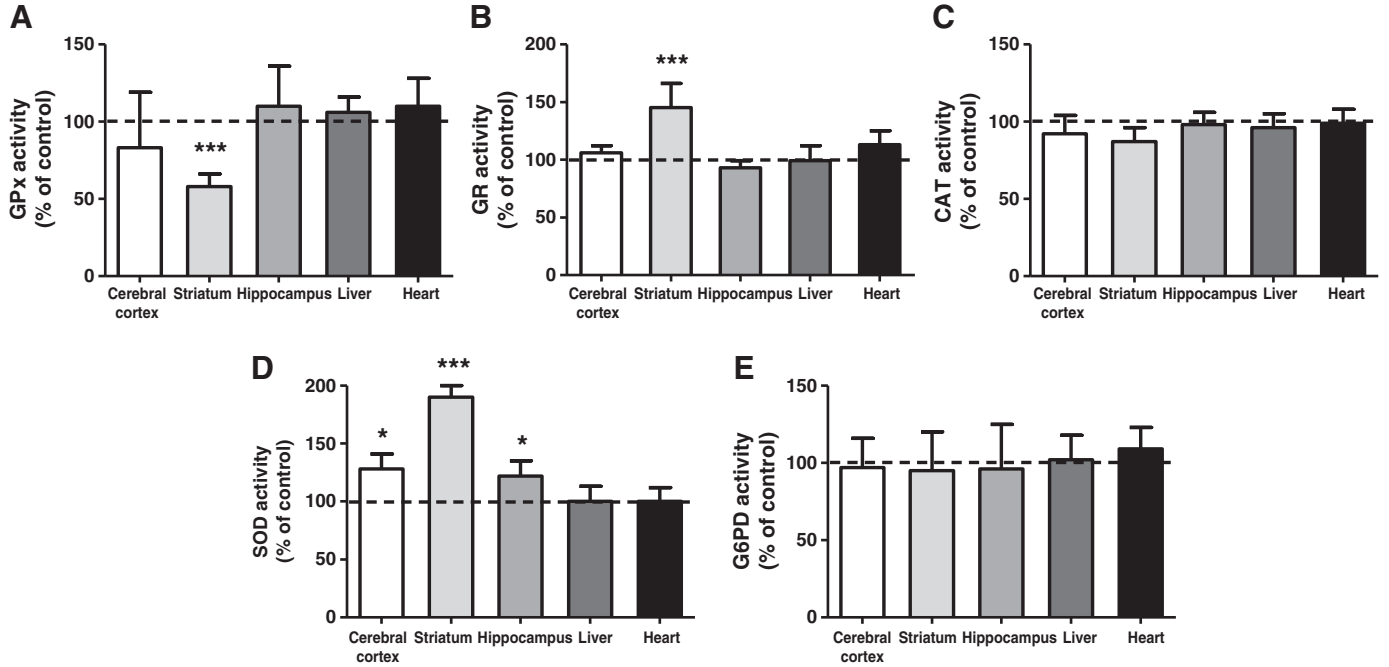

Fig. 4. Effect lysine (Lys, 8 μmol/g) intraperitoneal administration on the activity of the antioxidant enzymes glutathione peroxidase (GPx; A), glutathione reductase (GR; B), catalase (CAT; C), superoxide dismutase (SOD; D) and glucose-6-phosphate dehydrogenase (G6PD; E) in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: [A: 4.28 ± 1.41]; [B: 20.10 ± 0.63]; [C: 4.93 ± 0.59]; [D: 9.91 ± 1.21]; [E: 6.59 ± 1.15]; [U/mg protein]). ***$P<0.001$, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

effective scavenger of free radicals, a protector of thiol groups and a cofactor of GPx, α-tocopherol and melatonin [48,49], it is concluded that Lys overload reduced the brain tissue antioxidant defenses in the *Gcdh*$^{-/-}$ mice model. Furthermore, since a disruption of GSH system homeostasis may result in oxidative injury in sensitized neurons [50,51], it is presumed that our findings of reduced GSH levels in striatum and cerebral cortex caused by Lys administration in *Gcdh*$^{-/-}$ mice may be at least in part involved in the brain abnormalities observed in Lys-treated *Gcdh*$^{-/-}$ mice [4,32,33,52].

Furthermore, GPx activity was decreased and GR activity increased in the striatum, whereas SOD activity was augmented in all brain structures evaluated of the Lys-treated *Gcdh*$^{-/-}$ animals. We presume that induction of the expression of GR and SOD at the gene level might have taken place as a compensatory mechanism in response to increased formation of reactive species. This hypothesis is base on the observations that reactive oxygen species have previously been shown to regulate the expression of numerous genes, including those encoding antioxidant enzymes, via specific signaling mechanisms [47,53,54].

It must be considered that the brain has low activity of antioxidant enzymes and reduced content of non-enzymatic antioxidants [47], implying a lower capacity to react against free radicals compared with other tissues. The brain has also a high rate of oxidative metabolism coupled to ROS production, high amount of iron and greater peroxidation potential because of its elevated content of polyunsaturated fatty acids [46,55] that makes this tissue more vulnerable to increased formation of reactive species [55].

Since oxidative stress is caused by the imbalance between pro-oxidants and anti-oxidant mechanisms [46], our present data strongly indicate that acute Lys surplus induces in vivo oxidative stress in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice. It must be emphasized here that reactive oxygen species are capable to oxidize different molecules, including lipids, proteins and DNA, representing a key event in the pathogenic cascade leading to apoptotic (under chronic oxidative stress) or necrotic (under acute oxidative insults) cell death [56–58].

Interestingly, oxidative stress accompanies inflammatory processes [59–61] and patients with GA I frequently develop striatal damage during and after infections that are associated with inflammation and with a potential increase of tissue concentrations of GA and 3OHGA [18,62,63]. Furthermore, astroglyosis, a characteristic feature of neuroinflammation, is a common histopathological finding in the *Gcdh*$^{-/-}$ mice following exposure to chronically increased dietary Lys intake [33]. It has been also demonstrated that oxidative stress impairs the intrinsic cell potential, leading to proinflammatory and proapoptotic signals and creating a vicious circle between oxidative stress and neuroinflammation [64–66].

Our results showing that *Gcdh*$^{-/-}$ animals receiving acute Lys surplus resulted in 40% increase of GA in the brain, but not elevation of 3OHGA, suggest that probably GA induced oxidative damage and reduced the antioxidant defenses in these animals. It is emphasized that Lys easily crosses the blood brain barrier and is oxidized to GA in neural cells [18]. This is in agreement with the findings of Zinnanti and colleagues [34] demonstrating that high dietary Lys intake induces increased brain levels of GA which temporally parallels the mitochondrial abnormalities observed in striatum and cerebral cortex of 4-week-old *Gcdh*$^{-/-}$ mice. The significant body of in vitro and in vivo evidence showing that GA and 3OHGA provoke lipid and protein oxidation and reduce the antioxidant defenses in brain of 30-day-old rats reinforces the view that the accumulating metabolites in GA I, particularly GA, are mainly responsible for the brain damage in this disorder [21,27–29].

An intriguing issue in our results was why under basal levels no difference was encountered in the oxidative stress parameters between KO and WT mice. We feel that the inability to document any differences does not rule out the possibility that the chronic exposure of the *Gcdh*$^{-/-}$ mice to elevated GA and 3OHGA does lead to compensatory changes that we were unable to demonstrate, and that such compensatory mechanisms reduce their vulnerability to the chronic toxicity of the accumulated metabolites. Nonetheless, it is apparent that an acute Lys injection does provoke oxidative stress in the *Gcdh*$^{-/-}$ animals, suggesting that the rapid increase in GA (20-fold higher than in WT mice) overwhelms the antioxidant capacity of the brains of these mice.

Our observation that oxidative damage in the brains of *Gcdh*$^{-/-}$ mice was dependent on an increase in brain lysine and GA concentrations suggests that therapies aimed at reducing brain lysine uptake could be effective for treatment of GA I. Interestingly, it has been previously shown that arginine, which competes with lysine for brain transport, and glucose that also decreased brain lysine uptake, possibly by reduced utilization and compartmentalization, have beneficial effects in the mouse GA I model and in human patients [33,34,67,68].

5. Conclusions

In conclusion, the present results provide for the first time experimental evidence that oxidative stress is induced in vivo in the brain, and particularly in the striatum, of *Gcdh*$^{-/-}$ mice after acute Lys supplementation. It is therefore presumed that overproduction of reactive radicals is potentially deleterious to the CNS and may represent a relevant pathomechanism of brain injury in GA I and particularly during encephalopathic crises triggered by infections which are accompanied by high brain increases of GA concentrations. Thus, trials with antioxidants, especially those targeted to the mitochondria [69,70], considered the main source of reactive oxygen species, could be undertaken initially in the *Gcdh*$^{-/-}$ mice model and may potentially represent a novel adjuvant therapy together with Lys dietary restriction and L-carnitine supplementation in the treatment of GA I patients, in order to protect children from the devastating clinical sequelae of striatal damage.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

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