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Disturbance of redox homeostasis by ornithine and homocitrulline in rat cerebellum: A possible mechanism of cerebellar dysfunction in HHH syndrome

Ângela Zanatta [a], Carolina Maso Viegas [a], Anelise Miotti Tonin [a], Estela Natacha Brandt Busanello [a], Mateus Grings [a], Alana Pimentel Moura [a], Guilhian Leipnitz [a], Moacir Wajner [a,b,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

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ABSTRACT

Aims: Cerebellar ataxia is commonly observed in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, an inherited metabolic disorder biochemically characterized by ornithine (Orn), homocitrulline (Hcit) and ammonia accumulation. Since the pathophysiology of cerebellum damage in this disorder is still unknown, we investigated the effects of Hcit and Orn on important parameters of redox and energy homeostasis in cerebellum of young rats.
Material and methods: We determined thiobarbituric acid-reactive substance (TBA-RS) levels, carbonyl content, nitrate and nitrite production, hydrogen peroxide production, GSH concentrations, sulfhydryl content, as well as activities of respiratory chain complexes I–IV, creatine kinase, Na^+,K^+-ATPase, aconitase and α-ketoglutarate dehydrogenase.
Key findings: Orn and Hcit significantly increased TBA-RS levels (lipid oxidation), that was totally prevented by melatonin and reduced glutathione (GSH). We also found that nitrate and nitrite production was not altered by any of the metabolites, in contrast to hydrogen peroxide production which was significantly enhanced by Hcit. Furthermore, GSH concentrations were significantly reduced by Orn and Hcit and sulfhydryl content by Orn, implying an impairment of antioxidant defenses. As regards energy metabolism, Orn and Hcit provoked a significant reduction of aconitase activity, without altering the other parameters. Furthermore, Orn-elicited reduction of aconitase activity was totally prevented by GSH, indicating that the critical groups of this enzyme were susceptible to oxidation caused by this amino acid.
Significance: Taken together, our data indicate that redox homeostasis is disturbed by the major metabolites accumulating in HHH syndrome and that this mechanism may be implicated in the ataxia and cerebellar abnormalities observed in this disorder.

Introduction

The hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (MIM #238970) is an inherited disorder caused by a defect in the transport of ornithine (Orn) into the mitochondrial matrix due to mutations in the SLC25A15 gene, encoding the ornithine translocase 1 transporter previously termed ORNT1 also named ORC1 (Camacho et al., 1999; Fiermonte et al., 2003; Kim et al., 2012). The inability to import Orn from the cytosol into the mitochondria results in intramitochondrial Orn deficiency and a functional impairment of the urea cycle at the level of ornithine transcarbamoylase, with consequent hyperammonemia. Because the normal pathway for Orn catabolism proceeds via the intramitochondrial enzyme ornithine aminotransferase, cytosolic Orn accumulates resulting in hyperornithinemia. In the absence of intramitochondrial Orn, accumulating carbamoyl phosphate either condenses with lysine to form homocitrulline (Hcit), leading to homocitrullinuria, or is shunted through the cytosolic pyrimidine biosynthetic pathway leading to increased excretion of orotic acid and uracil in the urine (Korman et al., 2004).

HHH syndrome is clinically characterized by acute intermittent episodes of hyperammonemia accompanied by ataxia, vomiting, lethargy, and confusion. Besides cerebellar signs, patients also present movement disorders, dystonia, epilepsy, liver dysfunction and aversion to protein (Palmieri, 2008; Valle and Simell, 2001; Miyamoto et al., 2002; Kim et al., 2012; Filosto et al., 2012).

The central nervous system (CNS) pathology consists of demyelinization, atrophy and stroke-like lesions in the cerebral cortex and cerebellum, which have been ascribed to the toxic effects of ammonia and glutamine on the astrocyte, including changes in cellular bioenergetics, mitochondrial dysfunction, osmotic swelling, and alterations in glutamine–glutamate cycling (Gropman and Batshaw, 2004; Gropman, 2010;

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos No 2600 — Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5571; fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

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Tuchman et al., 2008; Braissant, 2010; Sofroniew and Vinters, 2010). However, it is unlikely that hyperammonemia is solely responsible for the pathophysiology of HHH syndrome since affected individuals who are diagnosed early and maintain good metabolic control (normal plasma ammonia levels) nonetheless develop progressive neurologic dysfunction years after their initial diagnosis. Therefore, chronic accumulation of Orn, HCit, orotic acid (Oro) and other metabolic factors cannot be ruled out as the contributing causes of the neurological symptoms seen in patients affected by HHH syndrome and investigation of the role of these accumulating metabolites on the CNS function will eventually lead to a better understanding of the relationship between the clinical features and the biochemical abnormalities of this disorder. In this context, recent studies revealed that Orn and Hcit disrupt mitochondrial homeostasis in vitro and in vivo in rat cerebral cortex (Amaral et al., 2009; Viegas et al., 2009, 2011).

It is of note that cerebellar ataxia and abnormalities are common findings in HHH syndrome, although the pathogenesis of the cerebellar damage in patients affected by this disorder is practically unknown. Therefore, in the present study we investigated the effects of Hcit and Orn on important biochemical parameters of oxidative stress and energy metabolism in cerebellum of young rats. We determined thiobarbituric acid-reactive substances (TBA-RS) (lipid peroxidation), carbonyl formation (protein oxidative damage), nitrate and nitrite formation (reactive nitrogen species) and hydrogen peroxide production (reactive oxygen species), reduced glutathione (GSH) concentrations and sulfhydryl content (non-enzymatic antioxidant defenses), as well as the activities of complexes I to IV (oxidative phosphorylation), aconitase and α-ketoglutarate dehydrogenase (citric acid cycle functioning), creatine kinase (intracellular energy transfer) and Na^+,K^+-ATPase (neurotransmission).

Experimental procedures

Animal and reagents

We used 30-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80–23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Hcit and Orn were dissolved on the day of the experiments in the buffer used for each assay to final concentrations in the incubation medium ranging from 0.1 to 5 mM, and the pH adjusted to 7.4.

Ethical statement

This study was performed in strict accordance with the EU Directive 2010/63/EU for Animal Experiments and approved by the Ethical Committee for the Care and Use of Laboratory Animals of HCPA. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data and to minimize the animal discomfort.

Preparation of cerebellum samples and incubation

Rats were sacrificed by decapitation without anesthesia, and the cerebellum was dissected, weighed and homogenized in 10 volumes (w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and pre-incubated for 1 h at 37 °C with Hcit or Orn. Controls did not contain these metabolites in the incubation medium. Immediately after pre-incubation, aliquots were taken to measure the oxidative stress parameters TBA-RS, carbonyl formation, sulfhydryl content, GSH levels, nitrate and nitrite production. In some experiments, antioxidants were co-incubated with supernatants at the following final concentrations: 1000 μM reduced glutathione (GSH), 1000 μM melatonin (MEL), 7.5 μM Trolox (TRO, soluble α-tocoferol), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 100 μM lipoic acid (LA). The doses of antioxidants used in the present study were selected according to the literature and to previous experiments demonstrating that these doses are capable of preventing oxidative damage and do not alter per se on the biochemical parameters analyzed (Leipnitz et al., 2008; Ribeiro et al., 2011; Moura et al., 2012; Tonin et al., 2012).

For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, the cerebellum was homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 $UI \cdot mL^{-1}$ heparin), pH 7.4. The homogenates were centrifuged at 800 *g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For the measurement of hydrogen peroxide (H_2O_2) release and the activities of aconitase and α-ketoglutarate dehydrogenase, mitochondrial fractions from cerebellum were prepared according to Rosenthal et al. (1987). For creatine kinase activity determination, the cerebellum was homogenized (1:10 w/v) in isosmotic saline solution and he homogenates used in the assay. For Na^+,K^+-ATPase activity, synaptic plasma membranes were prepared according to the method of Jones and Matus (1974). The cerebellum was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Then, the homogenates were added to a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 *g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation. These samples were pre-incubated for 30 min at 37 °C with Hcit or Orn. Controls did not contain these metabolites in the incubation medium.

We always performed the experiments using blanks in order to detect artifacts and validate our methodology. Whereas controls contained tissue preparations but not Orn and Hcit, some blanks were devoid of cerebellum preparations in the incubation medium supplemented by Orn or Hcit and served to detect interferences of the tested metabolites on the techniques utilized to measure the oxidative stress and bioenergetics parameters (results not shown).

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of pre-incubated cerebellum supernatants and centrifuged at 3000 *g* for 10 min. Three hundred microliters of the pre-incubated supernatants (containing approximately 0.3 mg of protein) were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/ mg protein.

Protein carbonyl content

Protein carbonyl formation, a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer (1994). Two hundred microliters of the aliquots from the pre-treated supernatants (containing approximately 0.3 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine