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

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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 dehydratase.

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 defences. As regards energy metabolism, Orn and Hcit provoked a significant reduction of aconitase activity, without altering the other parameters. Furthermore, Orn-elicted 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 demyelination, 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;
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), carboxyl formation (protein oxidative damage), nitrate and nitrite formation (reactive nitrogen species) and hydrogen peroxide (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-tocopherol), 500 μM N⁴-nitro- α-arginine methyl ester (L-NAME) and 100 μM lipic 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–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 U·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 (H₂O₂) 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 artifcats 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-dinitrophenylhydrazine 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).
(DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Samples were then precipitated with 600 μL 20% trichloroacetic acid and centrifuged for 5 min at 9000 g. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 10⁶ nmol/mL for aliphatic hydrazones.

Nitrates and nitrites production was measured according to Miranda et al. (2001). Two hundred microliters of vanadium chloride was added to the tube containing 200 μL of pre-treated supernatants (0.3 mg of protein) for complete reduction of nitrate to nitrite. Then, 200 μL of Griess reagent (a mixture of N-1-naphthylethlenediamine dihydrochloride and sulfanilamide) were added, and the tube was incubated for 30 min at 37 °C in a water bath in a dark room. The resulting pink-stained pigment was determined in a spectrophotometer at excitation and emission wavelengths of 535 and 587 nm, respectively, and slit widths of 5 nm. A calibration curve was performed using sodium nitrate (2.5–100 μM), and each curve point was subjected to the same treatment as supernatants. Nitrate and nitrite formation values were calculated as nmol/mg protein.

Mitochondrial hydrogen peroxide (H₂O₂) release

Mitochondrial preparations (0.2 mg protein·mL⁻¹) supported by 5 mM glutamate and 5 mM malate as substrates were incubated in standard reaction medium in the presence of 10 μM Amplex red and 1 U/mL horseradish peroxidase. The fluorescence was monitored during 500 s on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 5 nm. Antimycin A (0.1 μg/mL⁻¹) was added at the end of the measurements (Mohanty et al., 1997). Data were expressed as FAU.

Reduced glutathione (GSH) concentrations

GSH concentrations were measured according to Browne and Armstrong (1998). One volume of metaphosphoric acid was added to the pre-treated samples, which were centrifuged for 10 min at 7000 g. Then, 185 μL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of o-phthalaldehyde (1 mg/mL) were added to 30 μL of cerebral cortex supernatants (0.3–0.5 mg of protein). This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein.

Sulphhydril 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 (Aksenov and Markesbery, 2001). Briefly, 30 μL of 10 mM DTNB was added to an aliquot of 50 μL of pre-treated supernatants. This was followed by 30 min incubation at room temperature in a dark room. The sulphhydril content is inversely correlated to oxidative damage to proteins. Results were calculated as nmol TNB/mg protein.

Determination of the respiratory chain complexes activities

Mitochondrial respiratory chain enzyme activities (complexes I–III, II–III and IV) were measured in the supernatants with a protein concentration varying from 1.5 to 4.0 mg protein/mL. The activity of NADH:cytochrome c oxidoreductase (complexes I–III) was assessed as described by Schapira et al. (1990). The activities of succinate:DCIP-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complexes II–III) were determined according to the method of Fischer et al. (1985) and of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). The activities of the respiratory chain complexes were calculated as nmol/min/mg protein.

Activities of aconitase and α-ketoglutarate dehydrogenase complex

The activity of the enzyme aconitase was measured according to Morrison (1954), following the reduction of NADP⁺ at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004), with modifications. The incubation medium contained 50 mM K2PO4, 1 mM MgCl₂, 0.2 mM thiamine pyrophosphate, 0.3 mM DTT, 100 μM EGTA, 50 μM coenzyme A-SH, 250 μM α-ketoglutarate and 2 mM NAD⁺. pH adjusted to 7.35. The reduction of NAD⁺ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 e 466 nm, respectively.

Determination of creatine kinase activity

Creatine kinase activity was measured in the supernatants containing 0.4–1.2 μg of protein in a reaction mixture consisting of 60 mM Tris–HCl, pH 7.5, 7 mM phosphocreatine, 9 mM MgSO4 and 0.625 mM lauryl maltoside in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ATP. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962) with slight modifications (Schuck et al., 2002). The color was developed by the addition of 100 μL of 2% o-nitrophenol and 100 μL of 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were calculated as μmol creatine/min/mg protein.

Determination of Na⁺,K⁺-ATPase activity

The reaction mixture for the Na⁺,K⁺-ATPase assay consisted of 5 mM MgCl₂, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes containing approximately 3 μg of protein in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg⁡⁺-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na⁺,K⁺-ATPase activity was calculated by the difference between the two assays (Tsakiris and Dellikonstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme specific activity was calculated as nmol Pi released/min/mg protein.

Protein determination

Protein content was determined in cerebral cortex supernatants by the method of Lowry et al. (1951), using bovine serum albumin as a standard.
Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Only significant F 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.

Results

Orn and Hcit induce lipid peroxidation in rat cerebellum probably through reactive oxygen species formation

First, we investigated the in vitro effects of Orn and Hcit on TBA-RS levels in cerebellum of young rats. Fig. 1 shows that Orn [A: F(4,24) = 30.998; P < 0.001] and Hcit [B: F(4,24) = 8.897; P < 0.001] significantly increased TBA-RS levels in a dose-dependent manner [Orn: β = 0.919; P < 0.001; Hcit: β = 0.761; P < 0.001], indicating that these metabolites induce lipid peroxidation. We then evaluated the role of antioxidants on Orn-induced lipid oxidative damage. Cerebellar supernatants were co-incubated with 5.0 mM Orn and each of the antioxidants GSH (1500 μM), MEL (1000 μM), TRO (7.5 μM), L-NAME (500 μM) or LA (100 μM). Our results show that MEL [F(5,35) = 33.249; P < 0.001] and GSH [F(5,35) = 83.773; P < 0.001] were able to fully prevent Orn-induced TBA-RS levels increase, in contrast to TRO, L-NAME and LA, which did not alter Orn-induced lipid peroxidation (Fig. 4).

Orn and Hcit do not alter carbonyl formation in rat cerebellum

The next set of experiments was carried out to evaluate the in vitro effects of Orn and Hcit on carbonyl formation. We observed that neither Orn (control: 2.47 ± 0.89; 5 mM Orn: 2.69 ± 0.57; n = 6) nor Hcit (control: 4.24 ± 0.50; 5 mM Hcit: 4.65 ± 0.79; n = 6) significantly altered this parameter.

Orn and Hcit do not induce nitrate and nitrite production in rat cerebellum

We also assessed the effect of Orn and Hcit on nitrate and nitrite formation. These metabolites did not induce reactive nitrogen species generation in cerebellum (control: 1.51 ± 0.65; 5 mM Orn: 1.44 ± 0.51; 5 mM Hcit: 1.45 ± 0.58; n = 6).

Orn and Hcit provoke a reduction of non-enzymatic antioxidant defenses in rat cerebellum

The non-enzymatic antioxidant defenses were also assessed by investigating the effects of Orn and Hcit on GSH concentrations and sulfhydryl content in the cerebellum. We observed that Orn [F(4,24) = 42.691; P < 0.001] and Hcit [F(4,24) = 4.932; P < 0.05] significantly decreased GSH concentrations in a dose-dependent fashion [Orn: β = −0.662, P < 0.001; Hcit: β = −0.662; P < 0.001] (Fig. 2A). Furthermore, Orn decreased sulfhydryl content [F(4,24) = 3.013; P < 0.05] in a dose-dependent manner [β = −0.552; P < 0.01], in contrast to Hcit that did not alter this parameter (Fig. 2B).

Hcit increases hydrogen peroxide production in rat cerebellum

Next, we evaluated whether Orn or Hcit were able to alter the hydrogen peroxide (H$_2$O$_2$) production in the presence of glutamate and malate as substrates. We found that only Hcit significantly increased H$_2$O$_2$ production (Fig. 3).

The respiratory chain complexes I–IV activities are not altered by Orn and Hcit in rat cerebellum

We also tested the influence of Orn and Hcit on the activities of complexes I–II, II–III and IV of the respiratory chain. We verified that these activities were not altered by these metabolites (Table 1).

Orn and Hcit significantly inhibit aconitase activity in rat cerebellum

The effects of Orn and Hcit on α-ketoglutarate dehydrogenase and aconitase activities were also tested in mitochondrial preparations from cerebellum. We observed that these compounds did not change α-ketoglutarate dehydrogenase activity (control: 23.9 ± 5.27; 5 mM Orn: 26 ± 9.23; 5 mM Hcit: 21.2 ± 8.87; n = 6). However, Orn [F(4,16) = 5.667; P < 0.01] and Hcit [F(4,16) = 5.667; P < 0.05] markedly diminished aconitase activity in a dose-dependent manner [Orn: β = −0.819; P < 0.001; Hcit: β = −0.697; P < 0.01] (Fig. 5A). We then evaluated the role of antioxidants on Orn-induced aconitase activity inhibition. Cerebellar mitochondria were co-incubated with 5.0 mM Orn and GSH (1500 μM), MEL (1000 μM) or TRO (7.5 μM). Our results show that GSH [F(4,27) = 3.279; P < 0.05] was able to fully prevent Orn-induced aconitase activity decrease, whereas MEL and TRO did not alter this effect (Fig. 5B).

Orn and Hcit do not change creatine kinase and synaptic membrane Na$^+$, K$^+$-ATPase activities in rat cerebellum

We also verified that Orn and Hcit did not altered the activities of creatine kinase (control: 6.13 ± 1.11; 5 mM Orn: 7.7 ± 0.22; n = 6)
(control: 5.67 ± 2.22; 5 mM Hcit: 5.08 ± 1.46; n = 6) and synaptic membrane Na⁺, K⁺-ATPase (control: 804 ± 127; 5 mM Orn: 816 ± 57; n = 6) (control: 892 ± 225; 5 mM Hcit: 648 ± 374; n = 6).

Discussion

Ataxia and cerebellar atrophy are observed in HHH syndrome (Tsujino et al., 2000; Miyamoto et al., 2002; Korman et al., 2004; Filosto et al., 2012). Although the pathogenesis of the cerebellum damage is practically unknown, the CNS cellular pathophysiology in this disorder has been generally attributed to the toxic effects of ammonia (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. Therefore, it is conceivable that the other metabolites accumulating in HHH syndrome, i.e., Orn and Hcit, may be neurotoxic.

Therefore, we investigated the role of Orn and Hcit on important parameters of oxidative stress and energy homeostasis in rat cerebellum. We initially verified that Orn and Hcit significantly increased TBA-RS levels, reflecting an induction of malondialdehyde generation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007). Thus, it may be presumed that Orn and Hcit cause lipid peroxidation in cerebellum.

The next step of our investigation was to evaluate the effects of the classic antioxidants GSH, MEL, TRO, LA, SOD plus CAT and L-NAME on the lipid peroxidation induced by Orn. In this context, it is well known that GSH, MEL, TRO and LA are scavengers of the free radicals superoxide, hydroxyl and peroxyl, as well as of peroxynitrite. Besides its scavenging properties, GSH also protects protein bound sulfhydryl groups and eliminates hydrogen peroxide and lipid peroxide as a co-factor for the antioxidant enzyme glutathione peroxidase (Halliwell and Gutteridge, 2007). Thus, it may be presumed that Orn and Hcit cause lipid peroxidation in cerebellum.

The effect of nitric oxide synthase inhibitor L-NAME was finally evaluated in order to specifically determine the involvement of reactive nitrogen species.

We observed that Orn-induced lipid oxidative damage was totally prevented by the free radical scavengers GSH and MEL, suggesting that Orn provokes a prooxidant effect on membrane lipids from cerebellum probably by the hydroxyl and other radicals that are scavenged by

Fig. 2. Effect of ornithine (Orn) and homocitrulline (Hcit) on reduced glutathione (GSH) concentrations (A) and sulfhydryl content (B) in rat cerebellum. Values are means ± standard deviation for six independent experiments performed in triplicate and expressed as nmol/mg protein. *P < 0.05, **P < 0.001, compared to controls (Duncan multiple range test).

Fig. 3. Effect of ornithine (Orn) (A) and homocitrulline (Hcit) (B) on hydrogen peroxide production using 2.5 mM glutamate plus 2.5 mM malate as substrates in mitochondrial preparations from rat cerebellum. Antimycin (AA) (0.1 μg/mL) was added at the end of assays, as indicated. Traces are representative of four independent experiments and are expressed as fluorescence arbitrary units (FAU).
these antioxidants. Since nitrate and nitrite production was not altered by any of these metabolites, in contrast to hydrogen peroxide production, which was significantly enhanced by Hcit, it may be presumed that Orn- and Hcit-elicited oxidative damage through induction of reactive oxygen species generation. Interestingly, hydrogen peroxide gives rise to the hydroxyl radical by the Fenton reaction (Halliwell and Gutteridge, 2007).

We also found that GSH concentrations were decreased by Orn and Hcit, whereas sulfhydryl content was diminished by Orn. It should be emphasized here that GSH is the major naturally-occurring brain antioxidant (Halliwell and Gutteridge, 2007) and that sulfhydryl groups, which are found as protein-bound groups and as components of small molecules, represent an important redox antioxidant pool in the cellular defenses against oxidative stress (Hansen and Winther, 2009; Thomas et al., 1995; Requejo et al., 2010). Taken together, these findings indicate that the non-enzymatic antioxidant defenses are compromised by these metabolites in the cerebellum.

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue (Halliwell and Gutteridge, 2007), our present data strongly indicate that Orn and Hcit, the major amino acids accumulating in HHH syndrome, induce this deleterious cell condition in rat cerebellum. It should be also emphasized that the CNS has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 2007), a fact that makes this tissue more vulnerable to increased reactive species.

With respect to the parameters of energy metabolism, Orn and Hcit did not change the activities of the respiratory chain complexes I–IV, α-ketoglutarate dehydrogenase complex, creatine kinase and Na+,K+-ATPase. In contrast, we observed that Orn and Hcit decreased aconitase activity. In this particular, it is well reported that aconitase is a sensitive target of reactive oxygen species representing a parameter of oxidative stress (Liang et al., 2000; Patel et al., 1996; Tretter and Adam-Vizi, 2000; Tretter et al., 2005; Myers et al., 2010). In fact, Orn-induced decrease of aconitase activity was totally prevented by the antioxidant GSH, reinforcing the other findings demonstrating that the effects caused by Orn are mediated by reactive oxygen species. Furthermore, considering that aconitase is susceptible to oxidative inactivation, particularly by superoxide, resulting in the release of a labile Fe from its catalytic 4Fe–4S center and hydrogen peroxide
formation (Vasquez-Vivar et al., 2000; Myers et al., 2010), it is presumed that Hcit-induced inhibition of aconitase may be related to the increase of hydrogen peroxide. However, it should be noted that other reactive species are capable of inactivating aconitase (Gardner et al., 1997). Therefore, our findings demonstrating that Orn inhibited aconitase activity, but did not alter hydrogen peroxide levels, indicate that other reactive species are involved in Orn-induced aconitase inhibition.

Previous results obtained in cerebral cortex of rats showed a dual mechanism of brain damage caused by Orn and Hcit. It was revealed that these metabolites disturb energy homeostasis, by inhibiting the activities of respiratory chain complexes, CK and α-ketoglutarate dehydrogenase, as well as redox homeostasis, by causing protein and lipid oxidative damage and decreasing the antioxidant defenses (Amaral et al., 2009; Viegas et al., 2009, 2011). Our present results, demonstrating that oxidative stress is induced by Orn and Hcit in cerebellum with no disruption of energy dysfunction, indicate that oxidative damage and disturbance of antioxidant defenses probably represent the major mechanism of injury in this cerebral structure.

At the present we cannot determine the pathophysiological relevance of the present data since to our knowledge concentrations of Orn and Hcit in cerebellum or other brain structures were never measured in HHH syndrome. On the other hand, it should be considered that blood Orn concentrations may achieve 1 mM in affected patients and that some of our positive significant results were obtained at these doses (Palmieri, 2008; Valle and Simell, 2001). Although our present results better mimic an acute effect of the major metabolites accumulating in HHH syndrome, we cannot rule out that a persistent effect of elevated concentrations of Orn and Hcit may also induce a disruption of redox homeostasis in this disorder. Therefore, in case the present in vitro results are confirmed in vivo in animal models and in affected patients, it may be presumed that this pathomechanism may contribute to the cerebellar atrophy characteristic of HHH syndrome. It should be also noted that during episodes of metabolic decompensation characterized by encephalopathy and intense proteolysis, much higher concentrations of these amino acids take place therefore facilitating CNS injury (Camacho et al., 1999).

Conclusion

In conclusion, this is the first report showing that oxidative stress is elicited by Orn and Hcit in cerebellum of young rats. It is therefore presumed that, besides hyperammonemia, the major metabolites accumulating in HHH syndrome may contribute to the cerebellum abnormalities and symptoms found in patients affected by this disease possibly through reactive oxygen species attack. Furthermore, it is conceivable that a protein restricted diet with high caloric intake that reduce the risk of increased elevation of brain Orn and Hcit during catabolic states allied to the administration of antioxidants may represent a potential adjuvant therapy for patients affected by HHH syndrome.

Conflict of interest statement

The authors declare that there are no potential conflicts of interest.

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