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ARTICLE

Pleiotropic effects of 5-aminolevulinic acid in mouse brain

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Abstract: 5-Aminolevulinic acid (ALA) seems to be responsible for the neuropsychiatric manifestations of acute intermittent porphyria (AIP). Our aim was to study the effect of ALA on the different metabolic pathways in the mouse brain to enhance our knowledge about the action of this heme precursor on the central nervous system. Heme metabolism, the cholinergic system, the defense enzyme system, and nitric oxide metabolism were evaluated in the encephalon of CF-1 mice receiving a single (40 mg/kg body mass) or multiple doses of ALA (40 mg/kg, every 48 h for 14 days). We subsequently found ALA accumulation in the encephala of the mice. ALA also altered the brain cholinergic system. After one dose of ALA, a decrease in superoxide dismutase activity and a reduction in glutathione levels were detected, whereas malondialdehyde levels and catalase activity were increased. Heme oxygenase was also increased as an antioxidant response to protect the encephalon against injury. All nitric oxide synthase isoforms were induced by ALA, these changes being more significant for the inducible isoform in glial cells. In conclusion, ALA affected several metabolic pathways in mouse encephalon. Data indicate that a rapid response to oxidative stress was developed; however, with long-term intoxication, the redox balance was probably restored, thereby minimizing oxidative damage.

Key words: 5-aminolevulinic acid, heme metabolism, antioxidant defense system, cholinergic system, nitric oxide synthase.

Résumé : L'acide aminolévulinique (ALA) semble responsable des manifestations neuropsychiatriques de la porphyrie aiguë intermittente (PAI). Le but de ce travail était d'étudier l'effet de l'ALA sur différentes voies métaboliques dans le cerveau des souris afin d'accroitre notre compréhension de l'action de ce précurseur d'hème dans le système nerveux central. Le métabolisme de l'hème, le système cholinergique, le système de défense enzymatique et le métabolisme de l'oxyde nitrique ont été évalués dans l'encéphale de souris ayant reçu une seule (40 mg/kg de masse corporelle) ou de multiples doses (40 mg/kg, 14 jours) d'ALA. Les auteurs ont trouvé une accumulation d'ALA dans l'encéphale. L'ALA modifiait aussi le système cholinergique cérébral. Après l'administration d'une dose d'ALA, une diminution de l'activité de la superoxyde dismutase et une réduction des niveaux de glutathion étaient détectées, alors que les niveaux de malonaldéhyde et l'activité de la catalase étaient accrus. L'hème oxygénase, considérée comme réponse antioxydante, était aussi accrue afin de protéger cet organe du dommage. Toutes les isoformes de synthase d'oxyde nitrique étaient induites par l'ALA, ces changements étant plus significatifs dans les cellules gliales en ce qui concerne l'isoforme inductible. En conclusion, l'ALA a affecté plusieurs voies métaboliques dans l'encéphale. Les données indiquent qu'une réponse rapide au stress oxydant s'est développée; cependant, lors d'une intoxication à long terme, la balance redox était probablement rétablie, minimisant le dommage oxydant. [Traduit par la Rédaction]

Mots-clés : acide 5-aminolévulinique, métabolisme de l'hème, système de défense antioxydante, système cholinergique, synthase d'oxyde nitrique.

Introduction

Porphyrias are a group of inherited or acquired disorders of heme metabolism. All acute hepatic porphyrias display neurological symptoms, and the central nervous system (CNS) is involved during the acute attacks (Batlle 1997; Parera et al. 2003; Pischik and Kauppinen 2009; Besur et al. 2014). Heme biosynthesis is regulated by the first enzyme of the pathway, δ-aminolevulinic acid synthetase (ALA-S) (Huntera and Ferreira 2011). The product of this enzyme, 5-aminolevulinic acid (ALA), seems to be responsible for the neuropsychiatric manifestations of several porphyric disorders (Demasi et al. 1996; Batlle 1997; Felitsyn et al. 2008). Moreover, the pathophysiology of acute attacks in porphyrias could be related to low levels of heme as a cofactor for heme proteins such as nitric oxide synthase (NOS) and catalase (Herrick and McColl 2005).

ALA-generated reactive species have been shown to elicit oxidative lesions in synaptic membranes of the rat brain (Adhikari et al. 2006). The CNS is extremely sensitive to damage from free-radicals because it has a relatively small total antioxidant capacity (Ghosh et al. 2011; Schiavone et al. 2013).

We have previously reported that porphyrinogenic agents altered heme metabolism, the cholinergic and glutamatergic sys-

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tems, and the phase I drug metabolizing system, and also induced oxidative and nitrosative stress in mouse encephalon (Rodríguez et al. 2002; Rodriguez et al. 2005; Lavandera et al. 2007, 2009, 2011, 2015). These results could be attributed to a direct effect of toxins on the enzymes or to the disruption of heme biosynthesis, leading to deregulation of ALA-S and consequently to ALA accumulation.

The aim of this work was to study the effect of ALA on different metabolic pathways in mouse encephalon to enhance our knowledge about the action of this heme precursor on the CNS. To this end, heme metabolism (ALA-S and heme oxygenase (HO)), the cholinergic system (acetylcholinesterase (AChE) and butirylcholinesterase (BuChE)), the defense enzyme system (superoxide dismutase (SOD); catalase; glutathione peroxidase (GPx); glutathione reductase (GRed); reduced glutathione (GSH); and malondialdehyde (MDA)), and nitric oxide metabolism (nitric oxide synthase (NOS)) were evaluated in the encephalon of CF-1 mice treated with a single or multiple doses of ALA (Bechara 1996).

Materials and methods

Animals

Groups of 6–8 male albino CF-1 mice (25–30 g) were used to measuring the different parameters of each metabolic pathway investigated, and were maintained under controlled conditions and allowed free access to food (Purina 3; Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina) and water. Animals received humane care and were treated in accordance with the guidelines established by the Animal Care and Use Committee of the Argentinian Association of Specialists in Laboratory Animals (AADEALC).

Treatments

Acute treatment: animals received a single dose of 40 mg ALA/kg body mass (by intraperitoneal (i.p.) injection) and were sacrificed 24 h after the injection. Chronic treatment: animals received the same dose of ALA (40 mg/kg), every 48 h for 2 weeks, and were sacrificed 24 h after the last injection. Controls: Animals received the vehicle (NaCl, 0.9%) using the same protocols as described above, and they were sacrificed at the indicated times. All experiments were performed at the same time of the day.

Homogenate preparation

The encephalon (whole brain) of each mouse was scissored and immediately processed. Tissues from 2 mice were pooled and homogenized using a manual glass homogenizer in different solutions, depending on the parameter to be measured. For ALA-S determination, the homogenate was performed in a solution containing 0.9% NaCl, 0.1 mmol/L Tris–HCl (pH 7.4), 0.5 mmol/L EDTA (1:3, *w*/*v*) and it was used as enzyme source. AChE and BuChE activities were measured directly in the homogenate obtained from mouse encephalon in 0.1 mol/L sodium phosphate buffer (pH 8) (1:10 *w*/*v*). For the determination of MDA levels, encephala were homogenized (1:5 *w*/*v*) in 0.05 mol/L sodium phosphate buffer (pH 7.4) and analyzed immediately. An aliquot of this homogenate was used to measure catalase and GRed activities. For determining the other parameters we assayed, the encephala were homogenized in ice cold sucrose (0.25 mol/L; 1:3, *w*/*v*), and one aliquot was used to measure ALA levels. Then, the homogenate was centrifuged for 15 min at 1500*g*; the obtained supernatant was centrifuged again for a further 15 min at 10 000*g*, and the resulting pellet was washed once with the same solution used for homogenization and again centrifuged for 15 min at 10 000*g* (mitochondrial fraction). The supernatants from the first and the second centrifugations at 10 000*g* were pooled and centrifuged for 90 min at 105 000*g*; the pellet obtained was washed once with the buffer used for homogenization (microsomal fraction). The other parameters assayed were determined in the supernatants obtained by centrifugation at 10 000*g* (HO and cytosolic NOS activities, GSH levels) and 105 000*g* (SOD and GPx activities). Mitochondrial NOS (mtNOS) was measured in the mitochondrial fraction. Cholinestarases and HO activities were also evaluated from different areas of the encephalon, namely, the cortex, cerebellum, and hippocampus. Tissues were processed in the same manner as the whole brain was.

Assays

Biochemical assays

ALA levels were quantified following the technique of Mauzerall and Granick (1956). One-half millilitre of deproteinized homogenate was condensed with 0.05 mL of acethylacetone in Tris–HCl buffer (1 mol/L; pH 4.6), and the solution was heated to 100 °C for 10 min. After cooling to room temperature, an equal volume of modified Ehrlich's reagent (1 g of p-dimethylaminobenzaldehyde in 50 mL of glacial acetic acid : 70% perchloric acid; 21:4 *v*/*v*) was added, and the light absorption at 553 nm was determined after 8 min.

ALA-S activity was determined using the method of Marver et al. (1966). The reaction system contained, in a final volume of 1 mL, 75 mmol/L Tris–HCl buffer (pH 7.4), 0.1 mol/L glycine, 10 mmol/L EDTA, and 0.25 mL of crude homogenate. After 1 h at 37 °C, the reaction was stopped by adding 0.25 mL of 25% TCA. After centrifugation at 3000 rpm for 15 min, ALA was determined as was described above. One unit of ALA-S activity was defined as the quantity of enzyme that catalyzes the synthesis of 1 nmol of ALA per hour under standard conditions.

HO activity was measured as described by Tenhunen et al. (1970). The reaction mixture (1 mL) contained 0.1 mL of supernatant obtained by centrifugation at 18 000*g*, 34 μmol/L hemin, 90 μmol/L NADPH, and 90 mmol/L potassium phosphate buffer (pH 7.4). The formation of bilirubin was determined at 468 nm (molar extinction coefficient; 27.7 to 31.7) after 30 min at 37 °C.

SOD activity was assayed using the method of Paoletti et al. (1986), which is based in the inhibition of superoxide-driven NADH oxidation. The assayed aliquot (1 mL) consisted of 35 mmol/L sodium phosphate buffer (pH 7.4), 4 μmol/L NADH, a mixture of EDTA and $MnCl_2$ (0.03 mol/L – 0.015 mol/L, respectively), and 0.1 mL of different dilutions of enzyme extract. The decrease in the rate of NADH oxidation was measured at 340 nm for 5 min after adding 0.1 mL of 10 mmol/L β-mercaptoethanol. One unit of SOD was defined as the amount of SOD capable of inhibiting the rate of NADH oxidation, as measured in the control, by 50%.

Catalase activity was determined according to the methods of Chance and Maehly (1954) using 1 mL of a solution containing 0.05 mol/L potassium phosphate buffer (pH 7.0), 0.059 mol/L hydrogen peroxide (30%), and 0.1 mL enzymatic solution. The disappearance of peroxide was confirmed for 1 min at 240 nm with a spectrophotometer. One unit (enzyme activity) decomposed one nanomole of H_2O_2 per minute at 25 °C.

GPx activity was measured as described by Paglia and Valentine (1967). The reaction system (1 mL) contained 50 mmol/L sodium phosphate buffer (pH 7.4), 1 mmol/L EDTA, 1 mmol/L azide, 0.2 mmol/L NADPH, 1 mmol/L GSH, 0.01 U of GRed, and 0.1 mL of the enzymatic fraction. The conversion of NADPH to NADP was recorded at 340 nm for 5 min after initiating the reaction by adding 0.1 mL of 0.25 mmol/L H_2O_2. One unit of GPx was defined as the amount of the enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute under standard conditions.

GRed activity was quantified using the method of Pinto and Bartley (1969) following the reduction of glutathione disulfide (GSSG) to reduced glutathione by monitoring the oxidation of NADPH at 340 nm for 2 min. The incubation system contained, in a final volume of 1 mL, 83.5 mmol/L of sodium phosphate buffer (pH 7.4), 1.25 mmol/L EDTA, 3.125 mmol/L GSSG, 7.5 mmol/L NADPH, and 40 μL of enzymatic fraction. One unit of GPx was defined as the amount of the enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute under standard conditions.

The peroxidation index was evaluated by the formation of MDA and determined as thiobarbituric reactive species (TBARS) by the method of Niehaus and Samuelson (1968). To 0.5 mL of tissue homogenate, 1 mL of 2-thiobarbituric acid (0.375% *w*/*v* in 0.25 mol/L HCl and 15% TCA) was added, mixed thoroughly, and kept in a boiling water bath for 15 min. The precipitate was removed by centrifugation and the absorbance was measured at 535 nm.

GSH levels were evaluated according to the methods of Rossi et al. (1995). To 1 mL of acid deproteinized fractions were added 1.5 mL of EDTA (0.5 mg/mL in 0.08 mol/L sodium phosphate buffer (pH 8), and 0.1 mL of 5,5′dithio(bis)nitrobenzoic acid (4 mg/mL); after 15 min, we measured the absorbance at 410 nm.

NOS activity was measured in brain submitochondrial membranes and in the cytosolic fractions using a spectrophotometer to measure the oxidation of oxyhemoglobin to methemoglobin at 37 °C, as was previously described by Lavandera et al. (2011). The reaction system contained, in a final volume of 1 mL, 50 mmol/L potassium phosphate buffer (pH 7.4), 1 mmol/L $CaCl_2$, 100 μmol/L NADPH, 10 μmol/L dithiothreitol, 2 μmol/L Cu–Zn superoxide dismutase, 0.1 μmol/L catalase, 0.5–1.0 mg protein/mL, 25 μmol/L oxyhemoglobin, and 1 mmol/L L-arginine. Kinetics were followed at 577–591 nm (ε = (11.2 mmol·L^{-1})$^{-1}$·cm^{-1}) for 2 min. One unit of enzyme activity was defined as the quantity (in nanomoles) of enzyme that catalyzed the oxidation of oxyhemoglobin for 1 min under standard conditions. The specificity of methehemoglobin formation to NOS was confirmed by incubation with N^{ω}-nitro-L-arginine as the NOS inhibitor.

Cholinesterases

AChE and BuChE activities and muscarinic acethylcholinesterase receptor (mAChR) were quantified as was described by Rodríguez et al. (2002). The activities were determined using acethylthiocholine as substrate for both enzymes, and AChE was determined using ethiopropazine as the specific inhibitor of BuChE. The activity of BuChE was calculated by subtracting the AChE activity from the total enzyme activity measured in absence of inhibitor. The incubation system for total cholinesterase activity (AChE + BuChE) determination contained, in a final volume of 3.55 mL, 85 mmol/L sodium phosphate buffer (pH 8.0), 0.28 mmol/L DTNB, 0.54 mmol/L acetylthiocholine, and 0.05 mL of the enzymatic fraction. Absorbance at 412 nm was recorded for 5 min.

Protein concentration was estimated by either the procedure of Lowry et al. (1951) or Bradford (1976).

RNA extraction and Northern blot analysis

Total RNA was obtained with phenol–chloroform using the method of Chomczynski and Sacchi (1987) from the whole brain, and the mRNA expression was determined by Northern blot using specific probes. Total RNA (20 μg) was size fractioned by denaturing agarose gel electrophoresis and then transferred to a nylon membrane and left overnight. The crosslinking was done using an UV Stratalinker 1800 (120 mJ UV light). The membrane was prehybridized with 1 mL ULTRAhyb (Ambion) for each 10 cm^2 of membrane over a 4 h period. The cDNA probes were labeled with (^{32}P)dCTP by the Random Priming method using an oligolabelling kit (Bio-Labs). A 849 bp fragment of cDNA corresponding to 109 to 958 bases of the previously characterized rat HO-I mRNA (GeneBank accession number 14789657) was synthesized by PCR from mouse liver RNA using specific primers (forward primer, 5′-TCCACAGCCCGACAGCAT-3′; reverse primer, 5′-ATTCCCACTGCCACTGTTGC-3′). The cDNA probe used for ALA-S was a gift from Massayuki Yamamoto (Department of Biochemistry, Toyama Medical and Pharmaceutical University, School of Medicine, Toyama, Japan). The expression of the inducible form of HO (HO-I) and ALA-S mRNAs was normalized by comparison with the expression of 18S mRNA. The hybridized membrane was exposed to AGF radiographic film to visualize the bands. Quantitative analysis was performed with an image analyzer (LabScan version 3.00) and an image scanner (Amersham Pharmacia Biotech).

Western blot analysis

Analysis of the expression of the different isoforms of NOS was described in detail by Lavandera et al. (2011): anti-nNOS/NOS I (1:500 *v*/*v*; Santa Cruz Biotechnology) was used on the cytosolic and mitochondrial fractions, and anti-iNOS/NOS II (1:5 μg/mL; Upstate) on the cytosolic fraction. We detected a protein of 157 kDa that reacted with the anti-nNOS antibodies (amino terminus) in the cytosolic fraction, and a 144 kDa protein that reacted with the anti-nNOS antibodies in mitochondrial fraction (mtNOS). Gel loadings were normalized according to total protein content, and after immunoblotting, equal sample loading was confirmed by Coomasie blue staining, as described by Welinder and Ekblad (2011).

Immunohistochemical studies

Encephala were removed and fixed in 10% neutral-buffered formalin. At least 6 microtome sections of 3–5 μm were stained with haematoxylin–eosin. Immunohistochemistry was performed using the streptavidin–biotin–peroxidase complex system LSAB (DAKO). In brief, endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Microwave antigen retrieval was used (4 cycles of 5 min each in 0.1 mol/L citrate buffer using a 750 W microwave oven). Blocking solution (2% normal goat serum) was used before the specific antibody. The sections were incubated overnight at room temperature with anti-HO1 (1:400, Santa Cruz Biotechnology), anti-nNOS/NOS I (1:200; Santa Cruz Biotechnology), anti-iNOS/NOS II (1:200; Upstate), or anti-eNOS/NOS III (1:200; Santa Cruz Biotechnology). Control sections without primary antibody served as the control. The reaction was developed with 3,3′-diaminobenzidine (DAB), under microscopic control. Specimens were counterstained with 10% hematoxylin, dehydrated, and mounted. All photographs show the original magnification (250×). The different cell types (neurons and glia) were identified based on their morphology.

Statistical analysis

Data are the mean ± SD. Differences in mean values between the treatment and the control groups were evaluated using analysis of variance (ANOVA), and values for $p < 0.05$ were considered statistically significant.

Results

ALA accumulated in the encephalon affects heme metabolism

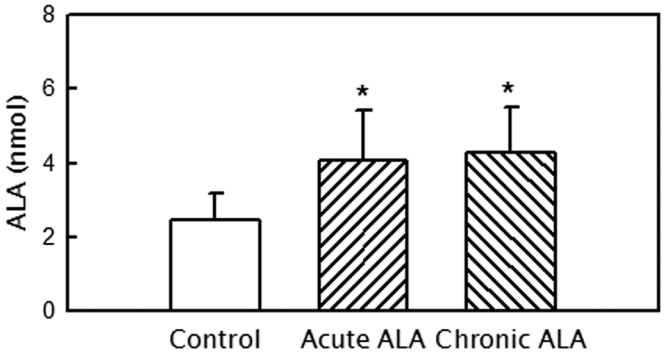
ALA levels were measured to determine whether there was an accumulation of this compound in the encephalon after ALA treatment (Fig. 1). The concentration of ALA increased by around 70% ($p < 0.05$) in comparison with the controls, in animals receiving ALA (both acute and chronic administration), without any difference related to the duration of the treatment. F1

To determine whether ALA affected heme synthesis regulation, the activity and expression of the regulatory enzyme ALA-S was measured (Fig. 2). No significant variations in ALA-S activity were produced (Fig. 2A), although mRNA levels of this enzyme were diminished by 80% and 60% in animals administered the acute or chronic ALA treatments, respectively (Fig. 2B). F2

Moreover, the effects on heme catabolism were evaluated measuring the activity and expression of HO, which is the first enzyme involved in the degradation of heme and is frequently induced under conditions of stress. HO activity was dramatically induced by ALA, being more pronounced after acute administration (445%; $p < 0.01$) and significantly different ($p < 0.05$) from that observed after chronic administration (50%; $p < 0.05$) (Fig. 3A). F3

To evaluate whether this induction was observed in all areas of the encephalon, HO activity was measured in the cortex, cerebellum, and hippocampus (Fig. 3B). No significant changes were ob-

Fig. 1. Levels of 5-aminolevulinic acid (ALA) after acute and chronic administration. Data are the mean ± SD for 6–8 male albino CF-1 mice; *, $p < 0.05$ indicates a statistically significant difference compared with the control group. The values from only one of the control groups were used because there were no significant differences among any of the controls after acute or chronic administration of the vehicle. Experimental details are described in the text.



served in the brain cortex. The activity of HO in the cerebellum showed significant induction in animals administered the acute (44%; $p < 0.05$) or chronic (98%; $p < 0.01$) treatments of ALA. In the hippocampus, a 115% ($p < 0.05$) increase in this enzyme activity was only found when ALA was chronically administered.

When encephalon mRNA levels of HO were quantified (Fig. 3C), no induction was observed after ALA treatment administered either acutely or chronically.

Immunohistochemical studies performed on the encephala of the controls and the treatment group receiving only one dose of ALA are shown in Fig. 4. A positive stain for HO-I in neurons and the glia were observed in the control animals (Fig. 4A). After acute ALA administration, the positive stain in the glia was slightly diminished (Fig. 4B). No alterations were observed after chronic administration of ALA (data not shown).

ALA altered the cholinergic system

The effects of ALA on the cholinergic system were evaluated by measuring AChE and BuChe enzymes (Fig. 5).

AChE activity was unchanged in the encephala of animals receiving the acute treatment of ALA (Fig. 5A), although it was increased by 40% ($p < 0.05$) after chronic administration of ALA. BuChE activity was reduced by 50% ($p < 0.05$) only in animals chronically treated with ALA (Fig. 5B).

Cholinesterases were also measured in different areas of the encephalon (Fig. 6). In the cortex, AChE activity (Fig. 5A) increased 41% ($p < 0.05$) after chronic administration of ALA, but there was no change in the treatment group that received one dose of ALA. Rather, acute ALA administration caused a 35% ($p < 0.05$) reduction in the activity of BuCHE in the cortex. In the cerebellum, enzyme activities were unchanged (Fig. 5B). In the hippocampus, the activity of AChE was also unaltered, but BuChE activity was increased by 58% ($p < 0.05$) in the group administered the acute treatment of ALA.

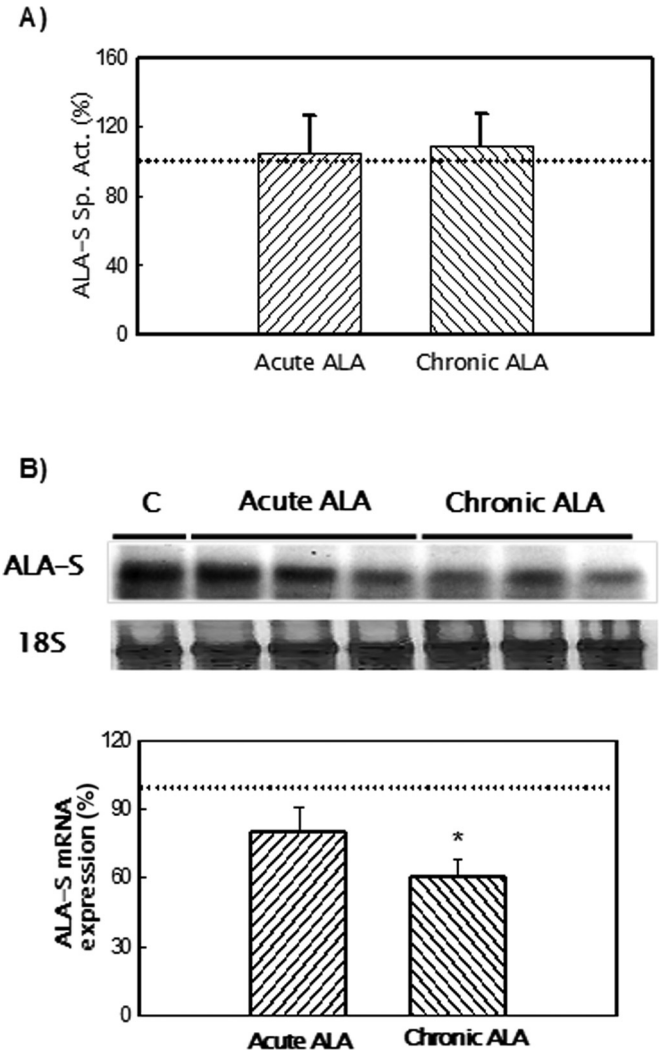
To complete our studies of the effects of ALA on the cholinergic system, mAChR levels were measured in the cortex, cerebellum, and hippocampus. No changes were observed in either the levels or affinity of mAChR (data not shown).

ALA-induced oxidative stress

To evaluate the oxidative stress status in mouse encephalon after the administration of ALA, we measured the activities of SOD, catalase, GPx, and GRed. GSH and MDA levels were also determined. Results are shown in Fig. 6.

SOD activity was reduced by 33% ($p < 0.05$) after both acute and chronic administration of ALA. Catalase activity was increased by

Fig. 2. (A) Activity and (B) mRNA expression of δ-aminolevulinic acid synthetase (ALA-S) after acute and chronic administration of ALA. (A) Data are the mean ± SD for 6–8 male albino CF-1 mice, and are expressed as a percentage of the control group (100%; broken line). Control value = 0.125 ± 0.084 nmol/mg ($n = 17$ mice); *, $p < 0.05$ compared with the acute and chronic treatment control groups, respectively. (B) Autoradiogram shows Northern blot analysis of ALA-S mRNA and 18S mRNA. Columns represent normalized signals in the control and treatment groups, which were quantified using an image analyzer. Values are expressed as a percentage of the control group (100%; broken line). Experimental details are described in the text.



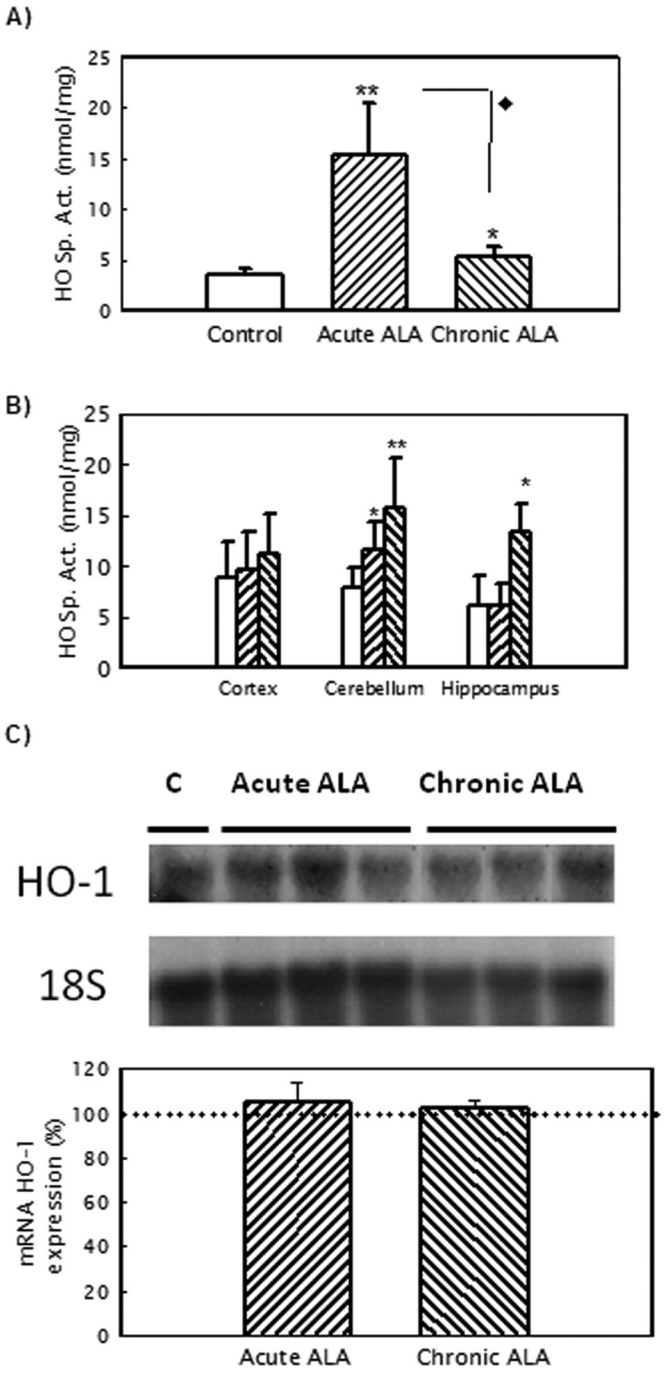
46% ($p < 0.05$) in the animals receiving the acute treatment with ALA, whereas there were no changes in the group administered the chronic treatment with ALA. GPx activity was unchanged after a single dose of ALA, but it was increased by 20% ($p < 0.05$) in the group chronically treated with ALA. No significant changes were detected in GRed activity in any of the test groups. GSH levels were diminished after acute (27%; $p < 0.01$) and chronic (23%; $p < 0.05$) treatment with ALA. MDA levels were increased by 30% ($p < 0.05$) after acute administration, but they were unchanged when ALA was chronically administered.

ALA altered the activity and expression of NOS

The effects of ALA administration on NOS activity in the cytosolic (nNOS and iNOS) and mitochondrial (mtNOS) subcellular fractions of encephalon are shown in Fig. 7.

Fig. 3. (A and B) Activity and (C) mRNA expression of heme oxygenase (HO) after acute and chronic administration of 5-aminolevulinic acid (ALA). Data in A and B are the mean ± SD for 6–8 male albino CF-1 mice treated with ALA; *, $p < 0.05$ and **, $p < 0.01$ compared with the control groups; ♦, $p < 0.05$ for the chronic administration group compared with the acute treatment group. The values from only one of the control groups were used because there were no significant differences among the controls after the acute or chronic administration of the vehicle. Experimental details are described in the text. (C) Autoradiogram showing Northern blot analysis of HO-I mRNA and 18S mRNA. Columns represent normalized signals in the control and treated animals and were quantified using an image analyzer. Values are expressed as a percentage of the control group (100%; broken line).

F8



Acute administration of ALA provoked a 35% reduction ($p < 0.05$) in cytosolic NOS activity, whereas mitochondrial activity was unaltered. After chronic administration of ALA, cytosolic activity remained diminished, whereas mitochondrial activity was enhanced (43%, $p < 0.05$) (Fig. 7A).

Taking into account the results obtained for NOS activity after acute and chronic administration of ALA, it was of interest to evaluate any differences in NOS expression and to study which isoform could be involved. Results are shown in Fig. 7B. nNOS expression was unchanged after acute or chronic administration of ALA, whereas mtNOS expression was reduced by ca. 60% ($p < 0.05$) in both cases. iNOS was undetectable by Western blot analysis.

Immunohistochemical studies showed nNOS-positive immunostaining in the Purkinje cells of the control animals (Fig. 8A), and increased levels in the animals receiving the acute treatment with ALA (Fig. 8B). When the iNOS staining was analyzed, control animals showed mild iNOS-positive staining, mainly in the neurons, and no staining in the glia (Fig. 8C), whereas in the acute ALA treatment group, iNOS staining appeared in the glia (Fig. 8D). eNOS was only detected in vessels, and it was unchanged after acute administration of ALA (data not shown). No differences in immunostaining among all of the NOS isoforms were detected after chronic administration of ALA (data not shown).

Discussion

ALA accumulation is a common feature observed in acute porphyrias (Batlle 1997). Several mechanisms have been postulated to explain the neuropsychiatric manifestations of these porphyrias, although the pathogenesis of acute attacks is yet unknown. In this work we have studied the effect of ALA administration on several metabolic pathways with the aim of demonstrating that it is not only one factor that triggers the neurological symptoms. This point is of great relevance for improving the effectiveness of treatments currently used to minimize the acute attacks of porphyria.

As we mentioned in the Introduction, ALA can cross the blood–brain barrier; although there are enough discrepancies to caution against making the assumption that increased levels of ALA in plasma would indicate that these levels should also be enhanced in the brain. Ennis et al. (2003) demonstrated that the brain is well-protected from changes in plasma levels of ALA by the very low blood–brain barrier permeability of ALA and by a saturable efflux mechanism present at the choroid plexus. Princ et al. (1994) demonstrated that ALA can be taken up by the cerebellum and cerebral cortex, and it can also be accumulated in cells. Our results showed a similar level of ALA after acute or chronic administration and, moreover, no differences in plasma levels were observed (data not shown). As was expected, ALA-S mRNA expression was diminished after acute and chronic ALA administration as a result of the feedback regulation of the heme pathway.

ALA is a source of ROS as a result of ALA enolization and the subsequent oxidation catalyzed by iron (Bechara 1996, Demasi et al. 1996). Brain is especially sensitive to ROS (Campese et al. 2004; Schiavone et al. 2013), and the oxidative stress produced by ALA could be one of the reasons for the neuropsychiatric syndrome of acute porphyrias (Halliwell 2001; Emanuelli et al. 2003; Adhikari et al. 2006). ALA induces lipid peroxidation in both the cerebellum and hippocampus, which are regions that exhibit neuropathological manifestations in AIP patients (Carneiro and Reiter 1998). Demasi et al. (1996) reported increased SOD activity after chronic ALA administration, whereas MDA levels were altered in sinaptosomal membranes but not in whole brain. Our findings demonstrated that when ALA was administered in an acute form, a decrease in SOD activity and GSH levels was detected whereas MDA levels and catalase activity were augmented. The fact that catalase activity was increased is probably due to a fast response to peroxide formation. These compounds would also act

Fig. 4. Expression of the inducible form of HO (HO-I) in CF-1 mice receiving acute administration of 5-aminolevulinic acid (ALA). Immunohistochemical studies: neuronal (yellow arrow) and glial cells (blue arrow) staining positive for HO-I. Magnification ×250 (original). Experimental details are described in the text. [Colour online.]

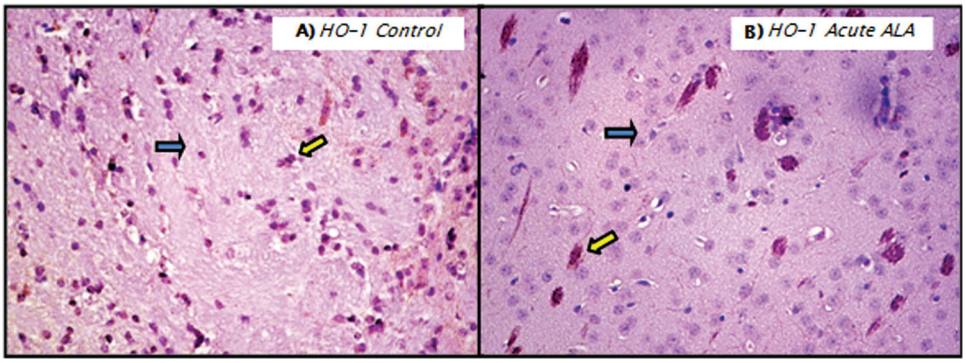


Fig. 5. (A) Acetylcholinesterase (AChE) and (B) butirylcholinesterase activities in the encephalon and other areas of the mouse brain after acute and chronic administration of 5-aminolevulinic acid (ALA). Data are the mean ± SD for 6–8 male albino CF-1 mice; *, $p < 0.05$ compared with the control groups. The values from only one of the control groups were used because there were no significant differences among the controls after the acute or chronic administration of the vehicle. Experimental details are described in the text.

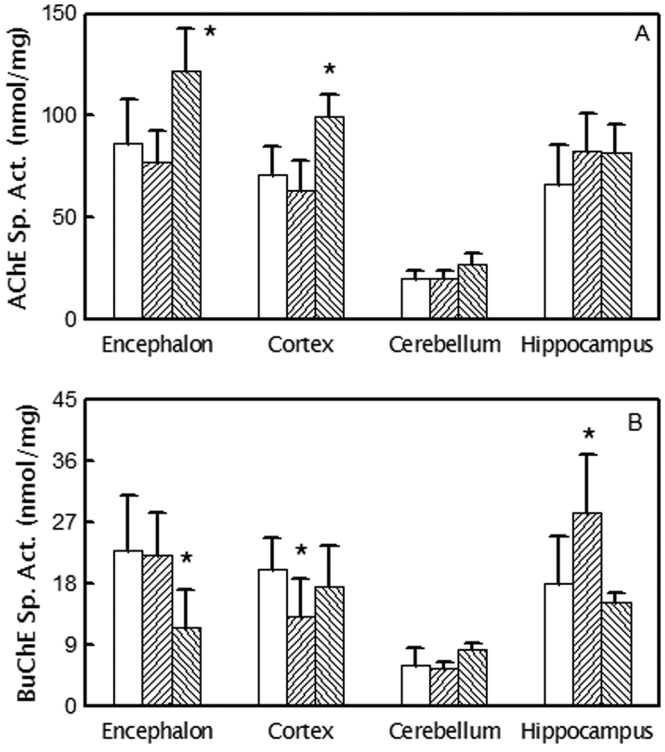
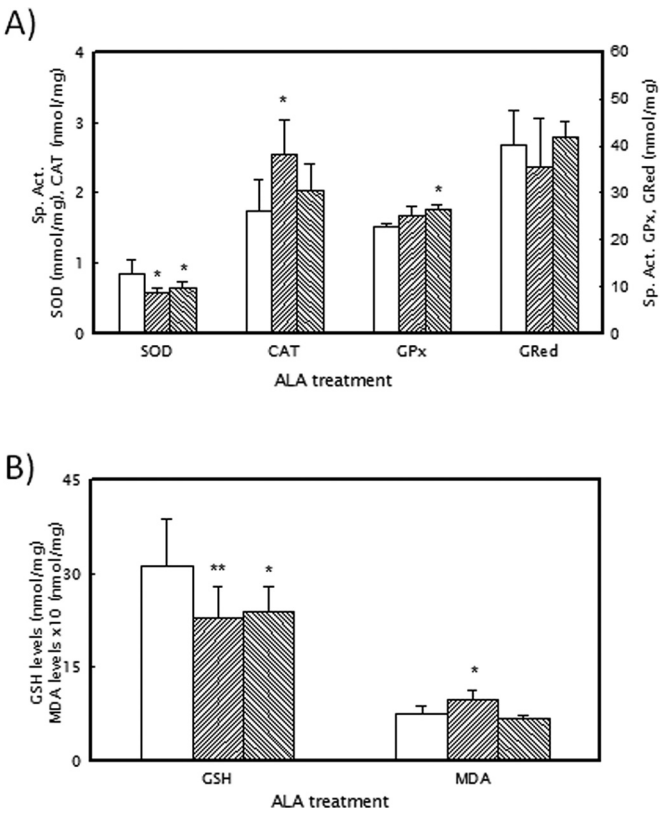


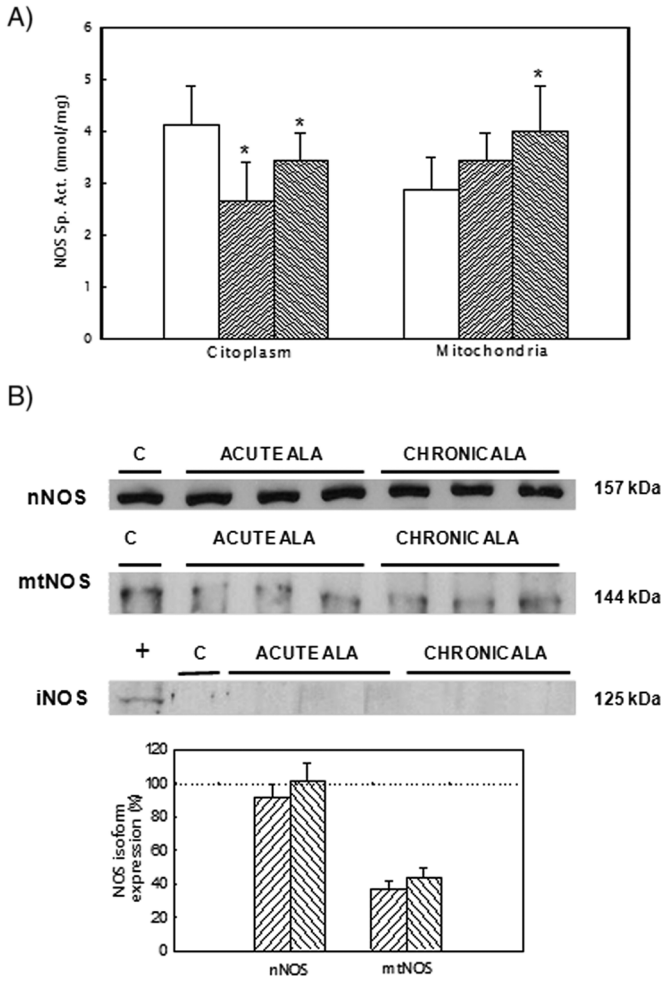
Fig. 6. Oxidative stress parameters in encephalon after acute and chronic administration of 5-aminolevulinic acid (ALA). (A) Activity of SOD, catalase, GPx, and GRed. (B) Levels of GSH and MDA. □, Control; ▨, acute ALA; ▧, chronic ALA. Data are the mean ± SD for 6–8 male albino CF-1 mice; *, $p < 0.05$ and **, $p < 0.01$ compared with the control groups. The values from only one of the control groups were used because there were no significant differences among the controls after the acute or chronic administration of the vehicle. SOD, superoxide dismutase; GPx, glutathione peroxidase; GRed, glutathione reductase; GSH, reduced glutathione; MDA, malondialdehyde. Experimental details are described in the text.



by inhibiting SOD activity. The presence of hydrogen peroxide would induce lipid peroxidation of membranes, as indicated by the high MDA levels observed. The low levels of GSH detected confirm that ROS are being generated. Some authors have suggested that stress may also cause GSH reduction, and that GSH-deficient animals are more vulnerable to stress-induced injury (Agarwal and Shukla 1999; Schulz et al. 2000; Dringen and Hirrlinger 2003). GSH is of vital importance for protecting tissues from oxidative damage; however, the mechanism of GSH depletion in stress is still unclear. A change in GPx activity in response to hydrogen peroxide, and GRed activity in response to GSH depletion shortly after ALA administration have not yet been reported. After chronic administration of ALA, GSH was maintained at low levels, but MDA content was normalized, and SOD activity stayed low. Catalase activity was restored, but it is worth noting that GPx activity was induced to protect cells from peroxide damage.

HO is a known oxidative-stress-inducible protein that plays a key role in heme catabolism, where heme, a potential prooxidant,

Fig. 7. (A) Activity and (B) expression of NOS after acute and chronic administration of 5-aminolevulinic acid (ALA). □, Control; ▨, acute ALA; ▧, chronic ALA. (A) Data are the mean ± SD for 6–8 CF-1 mice; *, $p < 0.05$ compared with the control groups. The values from only one of the control groups were used because there were no significant differences among the controls after the acute or chronic administration of the vehicle. (B) Autoradiogram showing Western blot analysis of NOS isoforms. NOS, nitric oxide synthase; C, control; +, positive control. Columns represent normalized signals in the control and treated animals, and were quantified using an image analyzer. Values are expressed as a percentage of the control group (100%; broken line). Gel loadings were normalized to the total protein content, and after immunoblotting equal sample loading was confirmed by Coomasie blue staining. Experimental details are described in the text.



is converted to bilirubin, an antioxidant (Maines 2000; Abraham and Kappas 2008). However, HO also produces other products, such as carbon monoxide, a signal transmitter, and free iron, another prooxidant. In the brain, HO markedly increases after heat shock, ischemia, or glutathione depletion (Maines 2000; Takeda et al. 2002). The existence in the SNC of a post-transcriptional control for other inducible genes was suggested (Quattrone et al. 2001) and this mechanism seems to be critical for brain functions. In addition to regulating the translation efficacy of HO-I mRNA, other types of post-transcriptional modifications have been proposed for this molecule (Bouton and Demple 2000) the might help with finely regulating HO-I mRNA expression.

The results presented here show a rapid induction of HO activity without any effect on mRNA expression in the encephalon. The activity induction varied depending on the area analyzed, being increased in the cerebellum and hippocampus after chronic administration of ALA. Immunohistochemical HO-I staining was reduced in the glia after acute administration of ALA, but HO-I-positive staining appeared in choroid (data not shown). Some reports have demonstrated that the choroid plexus, which is the site of the blood–cerebrospinal fluid barrier, selectively takes up ALA (Terr and Weiner 1983; Stummer et al. 1998; Novotny et al. 2000).

The changes that ALA administration causes both to the antioxidant system and the induction of HO reveal the triggering of a defense mechanism, as a consequence of the stress status provoked by this heme precursor, resulting from GSH depletion. An imbalance between cellular prooxidants and antioxidants is induced by high levels of ROS and reactive nitrogen species (Halliwell 2001; Mariani et al. 2005).

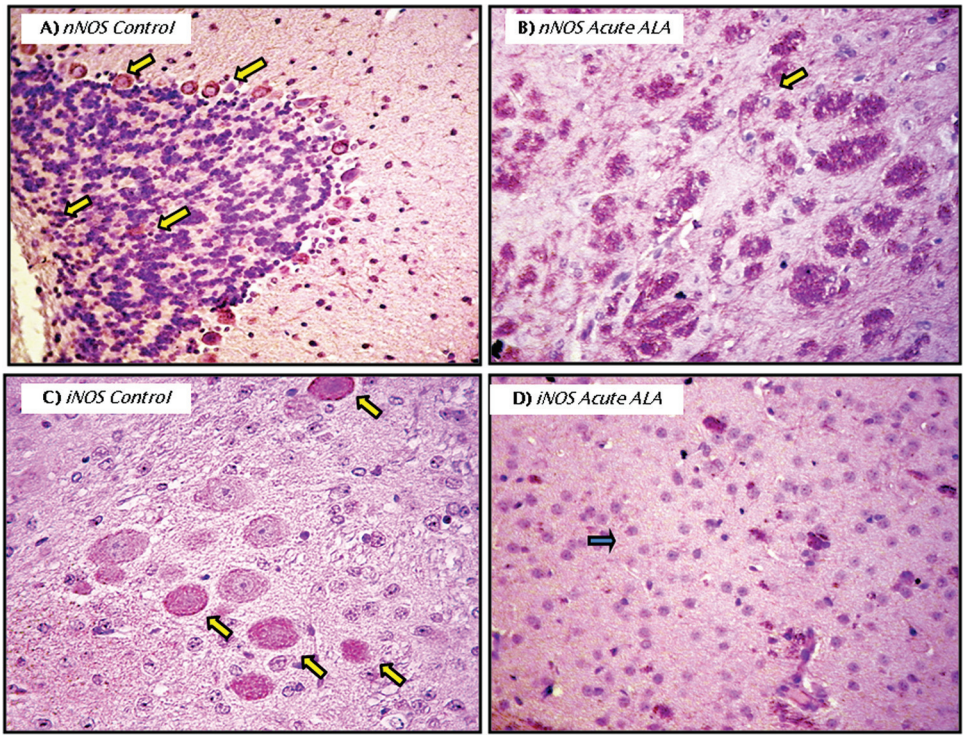
Nitric oxide is synthesized by nitric oxide synthase (NOS), a family with 4 major types: endothelial (eNOS), neuronal (nNOS), inducible (iNOS), and mitochondrial (mtNOS) (Gorren and Mayer 2007). They can be found in nearly all tissues and they can even co-exist in the same tissue. The relevance of NO in brain is determined by its physiological effects on neurons, glia, and the vascular system, and its involvement in neurodegenerative diseases (Guix et al. 2005; Pannu and Singh 2006). We have previously reported that NOS isoforms are induced by porphyrinogenic agents, being more significant in glial cells for iNOS (Lavandera et al. 2011; Buzaleh et al. 2012). In the present work, we observed that the acute and chronic administration of ALA diminished NOS activity in the cytosolic fraction of the brain tissue, whereas mitochondrial NOS activity was increased after chronic treatment with ALA, but was not changed in the same fraction after the acute treatment. Protein expression using Western blot technique demonstrated that mtNOS diminished after ALA treatments whereas nNOS was unchanged. Using immunostaining, iNOS expression could be detected, thereby revealing an increase in the glia and, as with HO-I, staining appeared in choroid (data not shown) after acute administration of ALA. Moreover, levels of nNOS staining also increased in Purkinje cells after acute administration of ALA. Purkinje neurons are a class of GABAergic neurons located in the cortex of brain cerebellum that play a fundamental role in controlling motor movement. The effects of ALA on the CNS have been explained by ALA binding to GABA(A) receptors, promoting receptor damage that may contribute to the neurological manifestations of AIP (Adhikari et al. 2006).

Besides the previously mentioned effects of ALA on the GABAergic system, ALA stimulates the release of glutamate and partially inhibits the re-uptake of glutamate (Emanuelli et al. 2003). We have previously reported that ALA also affects the glutamatergic system (Lavandera et al. 2009).

Altered activities and imbalance of dopaminergic, glutamatergic, GABAergic, and cholinergic systems are particularly involved in the pathogenesis of several neurodegenerative diseases, and several authors have remarked on the importance of the interaction between these neurotransmitters (Alberch et al. 1990; Xu et al. 2012; Deng and Dean 2013; Svob Strac et al. 2015). Moreover, the symptoms of acute attacks characteristic of acute porphyrias are similar to those observed during cholinergic crisis, which is why we examined the effects of ALA on the activities of the cholinesterases AChE and BuChE.

The cholinergic system was more noticeably affected by chronic ALA administration, and a differential response was observed in brain regions similar to that previously observed by the action of different porphyrinogenic drugs (Rodríguez et al. 2002). This differential response of the various areas of the brain had been reported. In this sense, Xu et al. (2001) observed that the hippocampus presented a lower vulnerability to the oxidative stress than the cerebral cortex after ALA administration for 15 days, whereas Carneiro and Reiter (1998) found, after 7 days of ALA treatment, significantly increased products of lipid peroxidation in cerebellum and

Fig. 8. NOS isoform expression in male albino CF-1 mice acutely administered 5-aminolevulinic acid (ALA). Immunohistochemical studies. Neuronal (yellow arrow) and glial cells (blue arrow) staining positive for NOS. Magnification ×250 (original). NOS, nitric oxide synthase. Experimental details are described in the text. [Colour online.]



hippocampus but not in the cortex, which are regions that exhibit neuropathological manifestations in AIP patients. So far, no explanation can be given for how the differences observed in the response of BuChE in cortex and hippocampus influence the neurological symptoms of acute porphyria, although results have indicated that very important biochemical changes would be occurring in the CNS because of ALA administration.

In conclusion, ALA produces a complex disorder involving deregulation of multiple metabolic pathways. Data indicate that a rapid response to oxidative stress was developed against the ROS induced by ALA treatment. However, with long-term intoxication (chronic adminstration), the redox balance was probably restored, thereby minimizing oxidative damage. We noted that the deregulation of the cholinergic system, together with the effects on other neurotransmitter systems observed previously, would contribute to the pathophysiology seen with acute porphyrias.

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References

Abraham, N.G., and Kappas, A. 2008. Pharmacological and clinical aspects of heme oxygenase. Pharmacol. Rev. **60**: 79–127. doi:10.1124/pr.107.07104. PMID: 18323402.

Adhikari, A., Penatti, C., Resende, R., Ulrico, H., Britto, L., and Bechara, E. 2006. 5-Aminolevulinate and 4,5-dioxovalerate ions decrease GABA(A) receptor density in neuronal cells, synaptosomes and rat brain. Brain Res. **1093**: 95–104. doi:10.1016/j.brainres.2006.03.103. PMID:16701578.

Agarwal, R., and Shukla, G.S. 1999. Potential role of cerebral glutathione in the maintenance of blood–brain barrier integrity in rat. Neurochem. Res. **24**: 1507–1514. doi:10.1023/A:1021191729865. PMID:10591399.

Alberch, J., Arenas, E., Sanchez Arroyos, R., and Marsal, J. 1990. Excitatory amino acids release endogenous acetylcholine from rat striatal slices: Regulation by gamma-aminobutyric acid. Neurochem. Int. **17**: 107–116. doi:10.1016/0197-0186(90)90074-4. PMID:20504609.

Batlle, A. 1997. Porfirias Humanas. Signos y Tratamientos. *In* Porfirias y Porfirinas. Aspectos clínicos, bioquímicos y biología molecular, Acta Bioquímica Clínica Latinoamericana. Suppl. No. 3, Chapter II, pp. 37–69.

Bechara, E. 1996. Oxidative stress in acute intermittent porphyria and lead poisoning may be triggered by 5-aminolevulinic acid. Braz. J. Med. Biol. Res. **29**: 841–851. PMID:9070373.

Besur, S., Hou, W., Schmeltzer, P., and Bonkovsky, H. 2014. Clinically important features of porphyrin and heme metabolism and the porphyrias. Metabolites, **4**(4): 977–1006. doi:10.3390/metabo4040977. PMID:25372274.

Bouton, C., and Demple, B. 2000. Nitric oxide-inducible expression of heme oxygenase-1 in human cells. Translation-independent stabilization of the mRNA and evidence for direct action of nitric oxide. J. Biol. Chem. **275**: 32688–32693. doi:10.1074/jbc.275.42.32688. PMID:11032845.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254. doi:10.1016/0003-2697(76)90527-3. PMID:942051.

Buzaleh, A.M., Meiss, R., Lavandera, J., Vallecorsa, P., Ruspini, S., and Batlle, A. 2012. Óxido Nítrico Sintasa y Hemo Oxigenasa en encéfalo de ratones tratados con anestésicos volátiles y otros agentes porfirinogénicos: Estudio inmunohistoquímico de la expresión proteica. Medicina, **72**(Suppl. II), 121, Abstract 241.

Campese, V.M., Ye, S., Zhong, H., Yanamadala, V., Ye, Z., and Chiu, J. 2004. Reactive oxygen species stimulate central and peripheral sympathetic nervous system activity. Am. J. Physiol. Heart Circ. Physiol. **287**: H695–H703. doi:10.1152/ajpheart.00619.2003. PMID:15277201.

Carneiro, R.C.G., and Reiter, R.J. 1998. Melatonin protects against lipid peroxidation induced by δ-aminolevulinic acid in rat cerebellum, cortex and hippocampus. Neuroscience, **82**(1): 293–299. doi:10.1016/S0306-4522(97)00262-5. PMID:9483521.

Chance, B., and Maehly, A. 1954. Assay of catalases and peroxidases. Methods Enzymol. **2**: 764–775. doi:10.1016/S0076-6879(55)02300-8. PMID:13193536.

Chomczynski, P., and Sacchi, N. 1987. Singe-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. **162**: 156–159. doi:10.1016/0003-2697(87)90021-2. PMID:2440339.

Demasi, M., Penalti, C., DeLucia, R., and Bechara, E. 1996. The prooxidant effect of 5-aminolevulinic acid in the brain tissue of rats: implications in neuropsy-

chiatric manifestations in porphyrias. Free Radic. Biol. Med. **20**: 291–299. doi:10.1016/0891-5849(95)02035-7. PMID:8720899.

Deng, C., and Dean, B. 2013. Mapping the pathophysiology of schizophrenia: interactions between multiple cellular pathways. Front Cell. Neurosci. **7**: 238. doi:10.3389/fncel.2013.00238. PMID:24348332.

Dringen, R., and Hirrlinger, J. 2003. Glutathione pathways in the brain. Biol. Chem. **384**(4): 505–516. doi:10.1515/BC.2003.059. PMID:12751781.

Emanuelli, T., Pagel, F., Porciuncula, L., and Souza, D. 2003. Effects of 5-aminolevulinic acid on the glutamatergic neurotransmission. Neurochem. Int. **42**: 115–121. doi:10.1016/S0197-0186(02)00074-8. PMID:12421591.

Ennis, S., Novotny, A., Xiang, J., Shakui, P., Masada, T., Stummer, W., et al. 2003. Transport of 5-aminolevulinic acid between blood and brain. Brain Res. **959**: 226–234. doi:10.1016/S0006-8993(02)03749-6. PMID:12493610.

Felitsyn, N., McLeod, C., Shroads, A.L., Stacpoole, P.W., and Notterpek, L. 2008. The heme precursor delta-aminolevulinate blocks peripheral myelin formation. J. Neurochem. **106**(5): 2068–2079. doi:10.1111/j.1471-4159.2008.05552.x. PMID:18665889.

Ghosh, N., Ghosh, R., and Mandal, S.C. 2011. Antioxidant protection: A promising therapeutic intervention in neurodegenerative disease. Free Radic. Res. **45**: 888–905. doi:10.3109/10715762.2011.574290. PMID:21615270.

Gorren, A., and Mayer, B. 2007. Nitric-oxide synthase: A cytochrome P450 family foster child. Biochim. Biophys. Acta, **1770**: 432–445. doi:10.1016/j.bbagen.2006.08.019. PMID:17014963.

Guix, F., Uribesalgo, X.I., Coma, M., and Muñoz, J.F. 2005. The physiology and pathophysiology of nitric oxide in the brain. Prog. Neurobiol. **76**: 126–152. doi:10.1016/j.pneurobio.2005.06.001. PMID:16115721.

Halliwell, B. 2001. Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. Drugs Aging, **18**: 685–716. doi:10.2165/00002512-200118090-00004. PMID:11599635.

Herrick, A.L., and McColl, K.E.L. 2005. Acute intermittent porphyria. Best Pract. Res. Clin. Gastroenterol. **19**: 135–249. doi:10.1016/j.bpg.2004.10.006.

Huntera, G.A., and Ferreira, G.C. 2011. Molecular enzymology of 5-Aminolevulinate synthase, the gatekeeper of heme biosynthesis. Biochim. Biophys Acta, **1814**(11): 1467–1473. doi:10.1016/j.bbapap.2010.12.015.

Lavandera, J., Batlle, A., and Buzaleh, A.M. 2007. Metabolization of porphyrinogenic agents in brain: involvement of the phase I drug metabolizing system. A comparative study in liver and kidney. Cell. Mol. Neurobiol. **27**: 717–729. doi:10.1007/s10571-007-9154-0. PMID:17676386.

Lavandera, J., Fossatti, M., Azcurra, J., Batlle, A., and Buzaleh, A.M. 2009. Glutamatergic system: another target for the action of porphyrinogenic agents. Cell. Mol. Biol. **55**(1): 23–28. PMID:19267998.

Lavandera, J., Batlle, A., and Buzaleh, A.M. 2011. Mice brain nitric oxide synthase response induced by anaesthetics and other porphyrinogenic drugs. Drug Metab. Lett. **5**: 25–29. doi:10.2174/187231211794455271. PMID:21198440.

Lavandera, J., Ruspini, S., Batlle, A., and Buzaleh, A.M. 2015. Cytochrome P450 expression in mouse brain: specific isoenzymes involved in phase I metabolizing system of porphyrinogenic agents in both microsomes and mitochondria. Biochem. Cell Biol. **93**(1): 102–107. doi:10.1139/bcb-2014-0088. PMID:25386744.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. 1951. Protein measurement with the folin–phenol reagent. J. Biol. Chem. **193**: 265–275. PMID:14907713.

Maines, M. 2000. The heme oxygenase system and its function in the brain. Cell. Mol. Biol. **46**: 573–585. PMID:10872744.

Mariani, E., Polidori, M.C., Cherubini, A., and Mecocci, P. 2005. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **827**: 65–75. doi:10.1016/j.jchromb.2005.04.023. PMID:16183338.

Marver, H.S., Tschudy, D.P., Perlroth, M.G., and Collins, A. 1966. Delta-aminolevulinic acid synthetase. I. Studies in liver homogenates. J. Biol. Chem. **241**: 2803–2809. PMID:4957991.

Mauzerall, D., and Granick, S. 1956. The occurrence and determination of delta-amino-levulinic acid and porphobilinogen in urine. J. Biol. Chem. **219**: 435–446. PMID:13295297.

Niehaus, W., and Samuelson, B. 1968. Formation of malonaldehyde from phospholipids during microsomal lipid peroxidation. Eur. J. Biochem. **6**: 126–130. doi:10.1111/j.1432-1033.1968.tb00428.x. PMID:4387188.

Novotny, A., Xiang, J., Stummer, W., Teuscher, N.S., Smith, D.E., and Keep, R.F. 2000. Mechanisms of 5-aminolevulinic acid uptake at the choroid plexus. J. Neurochem. **75**(1): 321–328. doi:10.1046/j.1471-4159.2000.0750321.x. PMID:10854277.

Paglia, D., and Valentine, W. 1967. Studies on the quantitative and qualitative characterization of erytrocyte glutathione peroxidase. J. Lab. Clin. Med. **70**: 158–169. PMID:6066618.

Pannu, R., and Singh, I. 2006. Pharmacological strategies for the regulation of inducible nitric oxide syntase: Neurodegenerative versus neuroprotective mechanisms. Neurochem. Int. **49**: 170–182. doi:10.1016/j.neuint.2006.04.010. PMID:16765486.

Paoletti, F., Aldinucci, D., Mocali, A., and Caparrini, A. 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissues extracts. Anal. Biochem. **154**: 536–541. doi:10.1016/0003-2697(86)90026-6. PMID:3089061.

Parera, V.E., De Siervi, A., Varela, L., Rossetti, M.V., and Batlle, A.M. 2003. Acute porphyrias in the Argentinean population: a review. Cell. Mol. Biol. (Noisy-le-grand), **49**(4): 493–500. PMID:12899439.

Pinto, R., and Bartley, W. 1969. The effect of age and sex on glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. Biochem. J. **112**: 109–115. doi:10.1042/bj1120109. PMID:4388243.

Pischik, E., and Kauppinen, R. 2009. Neurological manifestations of acute intermittent porphyria. Cell. Mol. Biol. (Noisy-le-grand), **55**(1): 72–83. PMID:19268005.

Princ, F., Juknat, A., and Batlle, A. 1994. Porphyrinogenesis in rat cerebellum. Effect of high delta-aminolevulinic acid concentration. Gen. Pharmacol. **25**: 761–766. PMID:7958739.

Quattrone, A., Pascale, A., Nogues, X., Zhao, W., Gusev, P., Pacini, A., and Alkon, D.L. 2001. Posttranscriptional regulation of gene expression in learning by the neuronal ELAV-like mRNA stabilizing proteins. Proc. Natl. Acad. Sci. U.S.A. **98**: 11668–11673. doi:10.1073/pnas.191388398. PMID:11573004.

Rodríguez, J.A., Buzaleh, A.M., Fossati, M., Azcurra, J., and Batlle, A.M. 2002. The effects of some porphyrinogenic drugs on the brain cholinergic system. Cell. Mol. Biol. (Noisy-le-grand), **48**(1): 103–110. PMID:11929041.

Rodriguez, J.A., Martinez Mdel, C., Gerez, E., Batlle, A., and Buzaleh, A.M. 2005. Heme oxygenase, aminolevulinate acid synthetase and the antioxidant system in the brain of mice treated with porphyrinogenic drugs. Cell. Mol. Biol. (Noisy-le-grand), **51**: 487–494. PMID:16309571.

Rossi, R., Cardaioli, E., Scaloni, A., Amiconi, G., and Di Simplicio, P. 1995. Thiol groups in proteins as endogenous reductants to determine glutathione-protein mixed disulphides in biological systems. Biochim. Biophys. Acta, **1243**: 230–238. doi:10.1016/0304-4165(94)00133-I. PMID:7873567.

Schiavone, S., Jaquet, V., Trabace, L., and Krause, K.H. 2013. Severe life stress and oxidative stress in the brain: From animal models to human pathology. Antioxid. Redox Signal. **18**(12): 1475–1489. doi:10.1089/ars.2012.4720. PMID:22746161.

Schulz, J.B., Lindenau, J., Seyfried, J., and Dichgans, J. 2000. Glutathione, oxidative stress and neurodegeneration. Eur. J. Biochem. **267**(16): 4904–4911. doi:10.1046/j.1432-1327.2000.01595.x. PMID:10931172.

Stummer, W., Stocker, S., Novotny, A., Heimann, A., Sauer, O., Kempski, O., et al. 1998. In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. J. Photochem. Photobiol. B, **45**: 160–169. PMID:9868806.

Svob Strac, D., Muck-Seler, D., and Pivac, N. 2015. Neurotransmitter measures in the cerebrospinal fluid of patients with Alzheimer's disease: A review. Psychiatr. Danub. **27**: 14–24. PMID:25751428.

Takeda, A., Itoyama, Y., Kimpara, T., Kutty, R.K., Abraham, N.G., Dwyer, B.E., et al. 2002. Role of heme catabolism in neurodegenerative diseases. *In* Heme Oxygenase in Biology and Medicine. *Edited by* N.G. Abraham. Kluwer Acad. Publ., N.Y., pp. 135–143.

Tenhunen, R., Marver, H.S., and Schmid, R. 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. J. Lab. Clin. Med. **75**: 410–421. PMID:4392030.

Terr, L., and Weiner, L.P. 1983. An autoradiographic study of delta-aminolevulinic acid uptake by mouse brain. Exp. Neurol. **79**: 564–568. doi:10.1016/0014-4886(83)90234-0. PMID:6822280.

Welinder, C., and Ekblad, L. 2011. Coomassie staining as loading control in Western blot analysis. J. Proteome Res. **10**: 1416–1419. doi:10.1021/pr1011476. PMID:21186791.

Xu, L., Sapolsky, R.M., and Giffard, R.G. 2001. Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. Exp. Neurol. **169**(2): 416–424. doi:10.1006/exnr.2001.7678. PMID:11358455.

Xu, Y., Yan, J., Zhou, P., Li, J., Gao, H., Xia, Y., and Wang, Q. 2012. Neurotransmitter receptors and cognitive dysfunction in Alzheimer's disease and Parkinson's disease. Prog. Neurobiol. **97**: 1–13. doi:10.1016/j.pneurobio.2012.02.002. PMID:22387368.