available at www.sciencedirect.com

SCIENCE DIRECT®

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

5-Aminolevulinate and 4, 5-dioxovalerate ions decrease $GABA_A$ receptor density in neuronal cells, synaptosomes and rat brain

Avishek Adhikari[a], *Carlos A.A. Penatti*[a], *Rodrigo R. Resende*[a], *Henning Ulrich*[a], *Luiz R.G. Britto*[b], *Etelvino J.H. Bechara*[a,*]

[a]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes 748, 05508-900 São Paulo, SP, Brazil*
[b]*Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil*

ARTICLE INFO

Article history:
Accepted 16 March 2006
Available online 15 May 2006

Keywords:
$GABA_A$ receptor
5-aminolevulinic acid
4,5-dioxovaleric acid
Oxidative stress
Intermittent acute porphyria
Lead poisoning

Abbreviations:
ALA, 5-aminolevulinic acid
CSF, cerebrospinal fluid
DOVA, 4,5-dioxovaleric acid
NF, neurofilament
SA, succinylacetone
SAME, succinylacetone methyl ester
GABA, γ-aminobutyric acid
RA, retinoic acid

ABSTRACT

Porphyrias are heme-associated metabolic disorders such as intermittent acute porphyria (IAP) and lead poisoning, where 5-aminolevulinate (ALA) accumulates. Effects of ALA on the CNS have been explained by ALA binding to $GABA_A$ receptors, followed by receptor lesions from oxyradicals and 4, 5-dioxovalerate (DOVA) generated from metal-catalyzed ALA oxidation by oxygen. We have characterized the effects of ALA and DOVA on $GABA_A$ receptor density in synaptosomes and neurons in vitro and also in brains of rats treated with ALA or succinylacetone methyl ester (SAME), a tyrosine catabolite derivative able to induce ALA accumulation. Radiolabeling assays revealed that following exposure to DOVA the concentration of synaptosomal GABAergic sites decreased by approximately 50%. Pretreatment with DOVA resulted in less $GABA_A$ receptor density in P19 and WERI cells and altered cell morphology. Furthermore, exposure to DOVA also induced a 5-fold increase in WERI cell mortality rate. Treatment with ALA resulted in loss of neuronal morphology and decrease of $GABA_A$ density in P19 neuronal cells. ALA and SAME treatment diminished the density of GABAergic receptors in the habenular complex and the parabigeminal nucleus of rat brain as studied by immunohistochemical procedures. Our results strongly suggest that ALA- and DOVA-promoted damage to $GABA_A$ receptors may contribute to the neurological manifestations of AIP and plumbism.

1. Introduction

5-Aminolevulinic acid (ALA) is a heme precursor that accumulates in hereditary porphyrias such as acute intermittent porphyria (Hindmarsch, 1986), chemical porphyrias (e.g., lead poisoning) (Gurer and Ercal, 2000), and tyrosinosis (Berger et al., 1983). It has been proposed that the neuropsychiatric manifestations of these diseases, which include mood disorders, aggressiveness, hallucinations, convulsions and seizures (Sassa and Kappas, 1983), are associated with

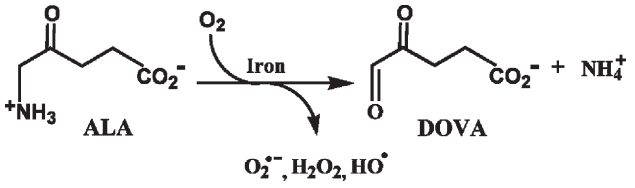
* *Corresponding author*. Fax: +55 11 38155579.
E-mail address: ebechara@iq.usp.br (E.J.H. Bechara).

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.03.103

accumulation of ALA (Kappas et al., 1995) due to enzymatic deficiencies in the heme biosynthetic pathway (Sassa and Kappas, 1983). Although the mechanism through which ALA is transported to the brain is uncertain, there are reports that accumulation of ALA in the brain may occur through passive diffusion between the blood/brain barrier (Ennis et al., 2003). Moreover, recent evidence for the existence of specific active transport of peptides (PEPT2) in cortical synaptosomes has been shown (Fujitaa et al., 2004). It is very likely that ALA is transported through that system, as for ALA has been shown to be a substrate for almost identical transporters (Ocheltree et al., 2004). In the choroid plexus, PEPT2 transporter contributes to the clearance of ALA from the CSF into the blood, thus keeping ALA concentrations low in the CSF (Ocheltree et al., 2004). This finding suggests that the brain has regular mechanisms to avoid ALA accumulation by actively keeping low levels of that heme precursor through several peptide transporters, in particular PEPT2, within the CNS. To date, some previous studies have shown that ALA is produced in the brain (Kappas et al., 1995; DeMatteis et al., 1981) and that cerebral cortex can accumulate ALA (Juknat et al., 1995). Thus, one can speculate that ALA levels in the compartmentalized brain tissue may actually reach millimolar range (Kappas et al., 1995), although further clinical studies are needed to provide better evidence of the brain concentrations of ALA in health and disease. Nevertheless, ALA increase in the brain can be viewed as a pathological sign and underscores its likelihood of playing a central role to the development of the neuropsychiatric manifestations in porphyrias.

It has been shown that ALA accumulation induces convulsions (Emanuelli et al., 2000), for ALA toxicity also involves glutamatergic pathways, since ALA irreversibly inhibits glutamate uptake in astrocytes through inhibition of the GLT-1 glutamate transporter (Emanuelli et al., 2003). Moreover, ALA damages $GABA_A$ receptors (Demasi et al., 1996), and acts as a GABA antagonist (Pierach and Edwards, 1978), besides stimulating glutamate release (Brennan and Cantrill, 1979). This suggests that ALA may be involved in neuronal cell death due to calcium influx (Choi, 1994).

ALA was suggested to behave as a pro-oxidant because it undergoes an iron-catalyzed oxidation by oxygen (Monteiro et al., 1986) at physiological pH with production of reactive oxygen species and 4,5-dioxovaleric acid (DOVA) (Scheme 1). Previous reports indicate that ALA may disrupt $GABA_A$ receptor function through oxyradicals formed during ALA oxidation (Demasi et al., 1996). ALA may indeed play a significant role in the neuropsychiatric manifestations of these syndromes, considering that it promotes release of iron from ferritin (Oteiza et al., 1995), thus exacerbating oxidative injury.



Scheme 1 – Aerobic oxidation of ALA catalyzed by iron with generation of reactive oxygen species and DOVA.

Considering the reactivity of DOVA towards biomolecules (Di Mascio et al., 2000), and that ALA accumulation would lead to DOVA formation, we hypothesize direct toxicity of DOVA in these syndromes. The aim of this work was to determine whether DOVA affects $GABA_A$ receptor density and to further study ALA and DOVA neurotoxicity in vitro and in vivo. These studies employed ALA and DOVA to investigate their effects on $GABA_A$ receptor density on WERI human retinoblastoma and differentiated neuronal cell line P19. Succinylacetone methyl ester (SAME) and ALA-treated rats were used for the in vivo studies. Succinylacetone is a potent inhibitor of 5-aminolevulinate hydrolyase (K_i = 0.03 μmol/L, EC 4.2.1.24), as previously demonstrated (Berger et al., 1983), and hence is able to induce ALA accumulation and mimic the metabolic state observed in the aforementioned illnesses.

2. Results

2.1. ^{3}H-muscimol binding in synaptosomes

To identify effects of DOVA on $GABA_A$ receptors, a binding assay was performed with synaptosomes using the $GABA_A$ agonist ^{3}H-muscimol (Fig. 1). Preliminary experiments were conducted to find the range of ^{3}H-muscimol concentrations in which nonspecific binding was lowest (0 to 25 nM) (data not shown). The pretreatment of synaptosomes with 2 mM DOVA for 30 min at 4 °C resulted in a 50% decrease of GABAergic binding sites. As the synaptosomal preparation was thoroughly washed five times to completely remove DOVA, the observed decrease in the number of GABAergic binding sites is not likely to arise from competition of DOVA with ^{3}H-muscimol. In fact, the change in the number of binding sites was used by chemical damage to the receptor, resulting in loss of ligand binding. Moreover, after DOVA treatment the shape of the curve, (as its derivative) did not change, thus it can be concluded that the binding affinity or dissociation constant (K_d) of the receptor did not change (Bylund and Yamamura, 1988).

The Rosenthal's plot obtained in this assay was nonlinear, indicating that DOVA may interact with more than one GABAergic site or with different membrane sites (Bylund and Yamamura, 1988).

2.2. $GABA_A$ receptor immunolabeling of WERI and P19 cells

In an attempt to determine whether ALA and DOVA-driven toxicity in synaptosomes to GABAergic receptors could be reproduced in $GABA_A$ receptor-expressing cell cultures, we chose human retinoblastoma WERI cells as in vitro models for $GABA_A$ receptor expression (Chou et al., 1999), by evaluating cell viability and morphology after exposure to ALA and DOVA.

Following confirmation of $GABA_A$ receptor expression, WERI cells were treated with ALA or DOVA (1 and 10 mM). Exposure to 1 mM ALA did not alter GABAergic receptor density in WERI cells. Treatment of WERI cells with DOVA (1 and 10 mM) and ALA (10 mM) altered cell morphology (Fig. 2), as the cells ceased to grow in the grape-like aggregates observed in control samples. Images of WERI cells after immunocytochemical staining (Fig. 3E) showed that exposure