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Research report

PTZ-induced seizures inhibit adenosine deamination in adult zebrafish brain membranes

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ABSTRACT

Adenosine exerts neuromodulatory functions with mostly inhibitory effects, being considered an endogenous anticonvulsant. The hydrolysis of ATP by ectonucleotidases is an important source of adenosine, and adenosine deaminase (ADA) contributes to the regulation of this nucleoside concentration through its deamination. In this study, we tested the effect of pentylenetetrazole (PTZ)-induced seizures on ectonucleotidase and ADA activities in adult zebrafish brain. Our results have demonstrated that PTZ treatments did not alter ectonucleotidase and ADA activities in membranes and soluble fraction, respectively. However, ecto-ADA activity was significantly decreased in brain membranes of animals exposed to 5 mM and 15 mM PTZ treatments (22.4% and 29.5%, respectively) when compared to the control group. Semiquantitative RT-PCR analysis did not show significant changes after the PTZ exposure on ADA gene expression. The decreased adenosine deamination observed in this study suggests a modulation of extracellular adenosine levels during PTZ-induced seizures in zebrafish.

1. Introduction

Epilepsy is one of the most common neurological diseases worldwide. This disorder is characterized by the occurrence of spontaneous and recurrent seizures that occur due to abnormal excessive and synchronous electrical activity of neuronal networks [22,20]. Adenosine is an endogenous modulator of brain functions that exhibits potent inhibitory effects on neuronal activity and has important anticonvulsant properties. These anticonvulsant properties are mediated mainly by A_1 receptors activation, which reduces the presynaptic neurotransmitter release and stabilizes the postsynaptic membrane potential [17,24]. Several studies have shown that this nucleoside is effective in suppressing the development and spread of seizures, in addition to preventing the epileptogenesis [21]. Adenosine treatments reduced the seizure frequency and prevented the progression of kindling epileptogenesis in animal models of epilepsy [33,41].

After exerting its effects through specific receptors, ATP is hydrolyzed by the cell-surface-located enzymes termed ectonucleotidases. Tri- and diphosphonucleosides may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine. Therefore, ectonucleotidases control ATP, ADP, AMP, and adenosine levels [47]. Although extracellular ATP is excitatory and might influence epileptogenesis, this nucleotide can be converted into adenosine, which has inhibitory effects [30]. Consequently, the control of ATP and adenosine levels through the pathway of ectonucleotidases may represent a key mechanism in the modulation of seizures and epileptogenesis (for review see [15]).

ADA is present both intracellularly and extracellularly and catalyzes the irreversible hydrolytic deamination of adenosine to inosine and ammonia. This enzyme is widely distributed in tissues and body fluids and shows different members in animal cells [23,35]. Rosemberg et al. reported ecto- and cytosolic-ADA activities in zebrafish brain [38]. Moreover, the different ADA-related gene expressions were identified in this teleost [37].

Several rodent protocols have reported the role of ectonucleotidases and ADA pathway in epilepsy. Late and prolonged changes in nucleotide hydrolysis were reported after status epilepticus

Abbreviations: ADA, adenosine deaminase; NTPDase, nucleoside triphosphate diphosphohydrolase; PTZ, pentylenetetrazole.

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induced by pilocarpine and kainate as well as PTZ-kindling protocols [9,10]. Moreover, adult rats submitted to one single seizure episode early in life presented enhanced extracellular ATP hydrolysis in hippocampal synaptosomes [16]. The administration of ADA activity inhibitors effectively reduced seizures in rodents [18,40]. Moreover, the adenosine deamination was significantly increased in the brain tissue of PTZ-kindled mice [27,28]. Thus, the investigation of the effects promoted by PTZ-induced seizures in distinct animal models may straightforward the knowledge related to acute seizure episodes and purinergic signaling.

The zebrafish is a small freshwater teleost which has been becoming widely used as a model to study the basis of epilepsy. Zebrafish larvae exposed to PTZ presented seizure behavioral and electrical discharges similar to those reported in rodent models. Also, these responses were suppressed by antiepileptic drugs [3,5]. Adult zebrafish exposed to the chemoconvulsant drugs PTZ, caffeine, and picrotoxin showed seizure-like behavioral responses and elevated whole-body cortisol levels [44]. PTZ-treated adult zebrafish showed an impairment in the passive avoidance responses, which was suppressed by the treatment with valproic acid [32]. Concerning the purinergic signaling, biochemical and molecular studies have already characterized NTPDase, ecto-5′-nucleotidase, and ADA in zebrafish brain [36,39,37,38]. Despite the importance of ectonucleotidases and ADA pathway in seizure occurrence has been already demonstrated in rodents, there is no evidence about the role of these enzymes after seizures in zebrafish, a non-mammalian model widely used for the screening of new pharmacological tools and therapies. Therefore, the aim of this study was to verify whether acute PTZ-induced seizures alter ectonucleotidases and ADA pathway in zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated. Fish were kept at 26 ± 2 °C under a 14–10 h light/dark cycle photoperiod and fed three times daily with commercial fish flake. The use and maintenance of zebrafish were according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 09/00117-CEUA.

2.2. Materials

Trizma base, ammonium molybdate, polyvinyl alcohol, malachite green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, and PTZ were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). TRIzol, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were from analytical grade.

2.3. In vitro assays

For *in vitro* experiments, PTZ (diluted in water) at 2.5, 5, 7.5, 10, 12.5, and 15 mM was directly added to the reaction medium, pre-incubated for 10 min with the brain membranes (for NTPDase, ecto-5′-nucleotidase, and ecto-ADA assays) or soluble fraction (for intracellular ADA) and were maintained throughout the enzyme assays. For the control group, the enzyme assays were performed in PTZ absence. Brain samples were obtained and the enzymes activities were tested as described for *in vivo* assays.

2.4. PTZ model

To induce seizures, zebrafish were individually exposed to 2.5, 5, and 15 mM PTZ in a 250 mL beaker. PTZ treatments were chosen based on previous study with zebrafish [3]. The animals were submitted to the treatment for 20 min and the seizure-like behavior was classified according each stage: stage I – increased swimming activity (2.5 mM), stage II – whirlpool swimming behavior (5 mM) and stage III – clonus-like seizures followed by loss of posture (fish falls to one side and remains immobile for 1–3 s) (15 mM) as previously reported for zebrafish larvae [3,5] and adults [44]. Control group was maintained in a 250 mL beaker with tank water for the same period and conditions as the PTZ-treated groups.

2.5. Preparation of soluble and membrane fractions

Brain samples were obtained as described previously [36,39,38]. Each independent experiment was performed using biological preparations consisted of a "pool" of five brains. First, zebrafish were cryoanaesthetized, euthanized, and brains were removed by dissection [43]. Samples were then further homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5′-nucleotidase assays zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously [4]. In brief, the homogenates were centrifuged at 800 × *g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40 000 × *g*. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at 40 000 × *g*. This freeze–thaw–wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

2.6. Ectonucleotidase assays

NTPDase and 5′-nucleotidase assays were performed as described previously [36,39]. Zebrafish brain membranes (3 μg protein for NTPDase and 5 μg protein for 5′-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) at a total volume of 200 μL. The samples were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min with 200 μL trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 mL of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi) [12]. The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi min^{-1} mg^{-1} of protein. In order to correct non-enzymatic hydrolysis of the substrates, controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.7. Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously [38]. The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 μL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [42]. In order to correct non-enzymatic hydrolysis of the substrates controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activity was expressed as nmol of NH_3 min^{-1} mg^{-1} of protein. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.8. Protein determination

Protein was measured by the Coomassie blue method [11] using bovine serum albumin as a standard.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of ADA-related genes *ada1*, *ada2.1*, *ada2.2*, and *adal* was analyzed by a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) assay. TRIzol® reagent (Invitrogen) was employed to isolate total zebrafish brain RNA in accordance with manufacturer instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values