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Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain

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ABSTRACT

Ethanol is a widely consumed drug that acts on the central nervous system (CNS), modifying several signal transduction pathways activated by hormones and neurotransmitters. The zebrafish is an experimental model for the study of human diseases and the use of this species in biochemical and behavioral studies on alcoholism and alcohol-dependence has increased recently. However, there are no data concerning the effects of chronic ethanol exposure on the purinergic system, where extracellular nucleotides act as signaling molecules. Purinergic signaling is controlled by a group of enzymes named ectonucleotidases, which include NTPDases and ecto-5′-nucleotidase already characterized in zebrafish brain. The aim of this study was to evaluate nucleotide hydrolysis by NTPDases and ecto-5′-nucleotidase after long-term ethanol exposure. Additionally, the gene expression patterns of NTPDases1–3 and 5′-nucleotidase were determined. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. There were no significant changes in ATP and GTP hydrolysis after all treatments. However, a decrease in ADP (46% and 34%) and GDP (48% and 36%) hydrolysis was verified after 7 and 14 days, respectively. After 7 and 14 days of ethanol exposure, a significant decrease in AMP hydrolysis (48% and 36%) was also observed, whereas GMP hydrolysis was inhibited only after 7 days (46%). NTPDase2_mv and NTPDase3 mRNA transcript levels decreased after 7 and 14 days, respectively. In contrast, ethanol increased NTPDase1, NTPDase2_mq, and NTPDase3 transcript levels after 28 days of exposure. NTPDase2_mg and 5′-nucleotidase gene expression was not altered. Therefore, the ectonucleotidase pathway may be a target of chronic ethanol toxicity and the regulation of purinergic system could play a key role in the neurochemical mechanisms underlying the effects of ethanol on the CNS.

1. Introduction

Alcohol abuse and alcoholism involve interactions among a number of neural mechanisms, including acute sensitivity to alcohol, development of tolerance, and dependence. The psychotropic effects of alcohol are mediated by complex interactions with ion channel systems and neurotransmitters (e.g. GABA, glutamate, dopamine, and noradrenaline) (Esel, 2006), leading to the typical behavioral effects on motor coordination, sensory perception, and cognitive performance (Fleming et al., 2001). Ethanol is able to disrupt purinergic signaling by inducing changes in ATP-activated P2X receptor function (Franke and Illes, 2006) and also by increasing the extracellular adenosine levels (Mailliard and Diamond, 2004).

ATP is a well-known co-transmitter together with classical transmitters in most nerves in the peripheral and CNS, although concentrations vary according to the tissue and species and in different developmental and pathophysiological circumstances (Burnstock, 2009a; Zimmermann, 2008). The many effects of ATP

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as a neurotransmitter are mediated by a series of nucleotide-selective receptors collectively named purinoceptors: P2 receptors (sensitive to ATP and ADP), and P1 receptors (sensitive to adenosine) (Burnstock, 2009b). P2 receptors are divided in two main families known as P2X and P2Y, which are ligand-gated ion channels and G protein-coupled receptors, respectively (Greig et al., 2008). Seven mammalian subtypes of P2X receptors (Gever et al., 2006) and eight subtypes of P2Y receptors have been recognized so far (Burnstock, 2006a,b). Adenosine can mediate different cellular functions by operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication (Fredholm et al., 2001).

After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which are functionally important because ATP metabolites act as physiological ligands for various purinergic receptors (Abbracchio et al., 2009). Thus, ectonucleotidases not only control the lifetime of nucleotide ligands but also produce ligands for additional P2 receptors and nucleosides by degrading or interconverting the originally released ligands (Zimmermann, 2006a). The ectonucleotidases include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5′-nucleotidase (Yegutkin, 2008; Zimmermann, 2006a). Individual enzymes differ in substrate specificity and product formation. E-NTPDases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-5′-nucleotidase. Alkaline phosphatases equally hydrolyze nucleoside tri, di and monophosphates. Four of the NTPDases are typical cell surface-located enzymes with their active site facing extracellularly (NTPDase1, 2, 3, 8). By contrast, NTPDases5 and 6 exhibit an intracellular localization and undergo secretion after heterologous expression. Meanwhile, NTPDases4 and 7 are located entirely intracellularly, facing the lumen of cytoplasmic organelles (Robson et al., 2006). ATP:ADP hydrolysis ratios for NTPDase1, 2, 3, and 8 are ~1–1.5:1, 10–40:1, 3–4:1 and 2:1, respectively (Kukulski et al., 2005; Yegutkin, 2008; Zimmermann, 2000).

Biochemical, molecular, and immunohistochemical studies have already described the ectonucleotidases in zebrafish (Rico et al., 2003; Ricatti et al., 2009; Senger et al., 2004). Bioinformatic and molecular studies reported and identified phylogenetically one ortholog gene for NTPDase1 (Senger et al., 2006), three paralog genes for NTPDase2 (Rico et al., 2006) and one gene for NTPDase3 (Appelbaum et al., 2007). Furthermore, the cloning and characterization of two P2X receptor subunits from zebrafish have been described (Boué-Grabot et al., 2000; Kucenas et al., 2003). Recently, two zebrafish A_{2A} and one A_{2B} genes were identified in developing embryos, and their expression was demonstrated in the CNS (Boehmler et al., 2009).

As a model for use in neuroscience, the zebrafish possesses numerous advantages including presenting a good balance between the simplicity and complexity of its organs and systems. The characterization of distinct neurotransmitter systems associated to the advances in the knowledge of the behavioral paradigms, make zebrafish an emergent vertebrate model for neurochemical studies (Gerlai et al., 2000; Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004; Ricatti et al., 2009; Cachat et al., 2011). Reports have described the use of zebrafish to study the reinforcing effects of drugs of abuse, such as morphine (Bretaud et al., 2007), cocaine (Darland and Dowling, 2001), and ethanol (Ninkovic and Bally-Cuif, 2006). Gerlai et al. (2000) evaluated the effects of acute ethanol treatment on zebrafish swimming behavior, group preference and pigment response. The ethanol directly mixed in the water can be absorbed by the blood vessels of the gill and the skin of the fish so that blood alcohol levels can reach equilibrium with the external alcohol concentration quickly and significant ethanol levels can be detected in the brain after a short period of exposure (Dlugos and Rabin, 2003). Furthermore, the zebrafish has been adopted as a genetic system for large-scale screening (Lockwood et al., 2004), and for the identification of genes that regulate the sensitivity and resistance to alcohol (Carvan et al., 2004). The adaptation of adult zebrafish after chronic exposure to ethanol has been demonstrated, such that tolerance to the acute effects of the drug develops (Dlugos and Rabin, 2003; Gerlai et al., 2006).

Behavioral parameters have been studied in the range of 0.25–1%, indicating a U-shaped dose response curve. These findings suggested that alcohol has a facilitatory effect at lower and inhibitory effect at higher doses (Gerlai et al., 2000). Recently, Dlugos et al. (2011) evaluated the effect of 0.5% ethanol exposure during 10 weeks on the swimming behavior from distinct zebrafish strains. Furthermore, our group demonstrated changes in NTPDase activities and expression and observed that 0.5% was the concentration that promoted the strongest inhibition on NTPDase activities after of acute ethanol exposure in zebrafish brain membranes (Rico et al., 2008). So far, however, there is no evidence about the effects promoted by chronic ethanol exposure on purinergic signaling parameters of adult zebrafish.

Therefore, the main goal of this study was to evaluate changes in adenine and guanine nucleotide hydrolysis promoted by ectonucleotidases in zebrafish brain after long-term ethanol treatment, as well as to investigate the gene expression pattern of NTPDases (1, 2, and 3) and 5′-nucleotidase.

2. Materials and methods

2.1. Zebrafish maintenance

Adult zebrafish of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50 L thermostated aquarium filled with continuously aerated unchlorinated water with Aquasafe® (Tetra, USA). The temperature was kept at 26 ± 2 °C under a 12-h light–dark controlled photoperiod, and the animals were fed twice a day until satiety with a commercial flake fish food (alcon BASIC®, Alcon, Brazil). The fish were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering and also the number of fish used. The Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under license number 477/05—CEP.

2.2. Chemicals

Ethanol (C_2H_6O) was purchased from Merck (Darmstadt, Germany). Trizma base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium chloride, magnesium chloride, and nucleotides (ATP, GTP, ADP, GDP, AMP, and GMP) were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.3. Ethanol treatment

For *in vivo* treatments, animals were introduced to the test aquariums (10 L) containing a solution of ethanol at 0.5% (v/v) and maintained in the test aquarium for 7, 14, and 28 days. Because a preliminary ethanol assay by infrared analysis ensured that there was no alteration in ethanol concentration every 48 h, the ethanol solution was replaced every two days. Immediately after the exposure, the fish were euthanized and membrane preparations were obtained.