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Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain

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Abstract

Alcohol abuse is a health problem throughout the world and alcohol consumption is linked to the occurrence of several pathological conditions. Acute ethanol administration exerts a variety of actions on the central nervous system (CNS). Zebrafish has been used as an attractive model system to investigate behavioral and neurochemical changes promoted by alcohol intoxication. Here we investigated the *in vitro* and *in vivo* effects promoted by ethanol and its metabolites on zebrafish brain acetylcholinesterase (AChE). There was a significant increase of AChE (33%) activity after acute 1% ethanol exposure. However, ethanol *in vitro* did not alter AChE activity. Acetaldehyde, the first metabolite of alcohol metabolism, promoted a dose-dependent decrease (15%, 27.5% and 46.5%) at 0.25%, 0.5% and 1%, respectively. Acetate, a product of acetaldehyde degradation, did not change AChE activity. Furthermore, the acute ethanol exposure was able to inhibit AChE transcripts at 0.5% and 1%. These findings suggest that the alterations on zebrafish AChE could reveal molecular mechanisms related to cholinergic signaling in alcoholism.

Keywords: Ethanol; Acetaldehyde; Acetylcholinesterase; Zebrafish; Alcoholism

1. Introduction

Alcohol abuse is a health problem throughout the world. Alcohol consumption is linked to the occurrence of many pathological conditions, such as various forms of cancer, liver disease, brain damage and fetal injuries during pregnancy (Quertemont et al., 2005). Furthermore, the acute ethanol effects impair motor coordination, sensory perception and cognition (Fleming et al., 2001).

Molecular mechanisms have been postulated in order to explain the effects promoted by ethanol, including damage induced by this alcohol and its metabolites, the production of oxygen reactive species (ROS) and oxidative stress (Sun and Sun, 2001). The major metabolic pathway for alcohol metabolism is through a two-step enzymatic process that requires nicotinamide adenine dinucleotide (NAD^+) as a co-factor. In the first step, the

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enzyme alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde, reducing NAD^+ in the process. In the second step, acetaldehyde is metabolized to acetate by the enzyme aldehyde dehydrogenase (ALDH), again reducing NAD^+ (Swift, 2003). Phylogenetic analysis of two zebrafish ADHs indicates that they share a common ancestor with mammalian ADHs (Reimers et al., 2004b). In addition, there are two minor oxidative pathways for metabolizing ethanol to acetaldehyde in brain, involving cytochrome P450 (CYP2E1) and the enzyme catalase. Although CYP2E1 is present in low levels in CNS, induction of this enzyme together with increased ROS production has been reported in rats after ethanol administration (Montoliu et al., 1995). Furthermore, in the presence of peroxides, the enzyme catalase can also oxidize ethanol to acetaldehyde and acetate (Swift, 2003).

Previous studies in zebrafish demonstrated that ethanol, after different exposure durations, leads to craniofacial abnormalities, cardiac malformations and developmental delays (Bilotta et al., 2004; Carvan et al., 2004; Reimers et al., 2004a). This species is used to study several neurobehavioral parameters, such as locomotor activity, learning, aggression and social interaction after acute ethanol exposure (Gerlai et al., 2000; Scalzo and Levin, 2004). Moreover, different zebrafish strains permit the investigation of genetic determinants involved in regulating the responses to ethanol (Dlugos and Rabin, 2003; Zon and Peterson, 2005).

Ethanol administration leads to an imbalance in different excitatory and inhibitory neurotransmitters such as the GABA, glutamate, dopamine, noradrenaline and acetylcholine (Hu et al., 1993; Esel, 2006). Two different types of cholinesterases are able to hydrolyze ACh: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8). It has been demonstrated that BuChE is not found on zebrafish genome and AChE is encoded by a single that was already cloned, sequenced and functionally detected in zebrafish brain (Bertrand et al., 2001). This enzyme can rapidly cleave ACh into choline and acetate and it has been described as a well-known biomarker for several environmental contaminants. Previous studies described that AChE can be affected by carbamate insecticides, methanol and heavy metals in different fish species, such as zebrafish and goldfish (Rico et al., 2006; Senger et al., 2006; Yi et al., 2006). In addition, it is already demonstrated that ethanol treatment can alter behavioral parameters, such as learning and memory, which is related with alterations on AChE activity in rat hippocampus (Pires et al., 2005).

Therefore, the aim of this study was to evaluate the *in vivo* and *in vitro* effects of ethanol on acetylcholinesterase activity in zebrafish brain, followed by the AChE gene transcriptional analysis after short-term ethanol treatment.

2. Methods

2.1. Animals

Adult zebrafish were obtained from commercial supplier (Delphis, RS, Brazil). All fish were acclimated to their new environment for at least 2 weeks in 50-L conditioned at $25 \pm 2\,^{\circ}C$ under natural light–dark photoperiod. They were used according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. The Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) approved the protocol under the number 477/05—CEP.

2.2. Chemicals

Ethanol (C_2H_6O; CAS number 64-17-5) and acetate ($C_2H_4O_2$; CAS number 127-09-3) were purchased from Merck (Darmstadt, Germany), and Acetaldehyde (C_2H_4O; CAS number 75-07-0) from Fluka (Switzerland). Trizma Base, EDTA, EGTA, sodium citrate, Coomasie Blue G, bovine serum albumin, acetylthiocholine, 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (St. Louis, USA).

2.3. In vivo and in vitro treatments

For acute treatment, fish were introduced in 20-L aquariums containing three different concentrations of ethanol (v/v) (0.25%, 0.5% and 1%). The animals were exposed for 1 h and the brains were dissected before performing biochemical and molecular analysis. For *in vitro* assays, ethanol, acetaldehyde and acetate were added to the reaction medium before the enzyme preincubation and maintained throughout the enzyme assays. The final concentration of ethanol, acetaldehyde and acetate were in the range of 0.25–1%.

2.4. Determination of AChE activity

A pool of five zebrafish brains was used to prepare each homogenate fraction. The brains were homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon–glass homogenizer. The rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB was determined as described previously (Ellman et al., 1961). Before the addition of substrate, samples containing protein (10 μg) and the reaction medium mentioned above were preincubated for 10 min at 25 °C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to deter-

mine the non-enzymatic hydrolysis of ACSCh. The linearity of absorbance towards time and protein concentration was previously determined. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. We performed four different replicate experiments.

2.5. Protein determination

Protein was measured using Coomassie Blue as color reagent (Bradford, 1976) and bovine serum albumin as a standard.

2.6. Molecular analysis

In order to identify AChE zebrafish orthologous genes, the mouse protein sequence (NP_571921) was used as query. NCBI Blast searches of GenBank yielded one AChE zebrafish sequence (CAC19790) previously described (Bertrand et al., 2001). Forward (5′-CCAAAAGAATAGAGATGCCATGG-ACG-3′) and reverse (5′-TGTGATGTTAAGCAGACGAGG-CAGG-3′) primers and the optimal conditions for RT-PCR were used according to Rico et al. (2006). The β-actin primers forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′-GCCGGACTCATCGTACTCCTG-3′) were used as described previously (Chen et al., 2004).

Total RNA was isolated from zebrafish brain using Trizol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/μL. cDNA species were synthesized with SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the suppliers. One microliter of RT reaction mix was used as a template for each PCR. PCR for AChE was performed in a total volume of 25 μL using 0.08 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). PCR for β-actin gene was performed in a total volume of 20 μL using 0.1 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). PCR were conducted at 1 min at 94 °C, 1 min at 60 °C (AChE) and at 54 °C (β-actin), and 1 min at 72 °C for 35 cycles. A post-extension cycle at 72 °C was performed for 10 min.

For each set of PCR, negative control was included. PCR products were analyzed on 1% agarose gel, containing ethydium bromide and visualized with ultraviolet light. The Invitrogen 1 kb ladder was used as molecular marker and normalization was performed employing β-actin as a constitutive gene. The band intensities were measured by optical densitometry analysis and the enzyme/β-actin mRNA ratios were established for each treatment using the Kodak 1D Image Analysis Software.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as mean ± S.D. A Duncan multiple test range as post hoc was performed,considering $P < 0.05$ as significant.

3. Results

The acute ethanol treatment was evaluated on AChE activity in zebrafish brain. The experiments were performed after 1 h *in vivo* ethanol exposure at varying concentrations (0.25%, 0.5% and 1%). Ethanol, at 0.25% and 0.5%, did not alter AChE activity in zebrafish brain. However, this enzyme activity was significantly increased (33%) at 1% ethanol when compared to the control group (Table 1). On the other hand, the *in vitro* ethanol treatments did not promote any significant changes on zebrafish brain AChE activity when tested at 0.25%, 0.5% and 1% (Table 1).

In order to verify whether the metabolites acetaldehyde and acetate could influence AChE activity, these compounds were added directly to reaction medium at 0.25%, 0.5%, and 1%. Acetaldehyde presented a significant effect on AChE, inhibiting *in vitro* ASCh hydrolysis (15%, 27.5% and 46.5%) at 0.25%, 0.5%, and 1%, respectively (Table 1). Acetate was not able to promote any alteration on the AChE activity in the same concentrations mentioned above (Table 1). Since acetate can mediate ethanol effects (Israel et al., 1994), we performed experiments to evaluate the *in vivo* effects of acetate on AChE activity. The aquariums were treated with sodium acetate (ranging from 0.1% to 1%) and there were no significant changes on AChE activity (data not shown).

Table 1
In vivo ethanol treatments and *in vitro* effects of ethanol, acetaldehyde and acetate on AChE activity in zebrafish brain

Concentration (%)	*In vivo*	*In vitro*		
	Ethanol	Ethanol	Acetaldehyde	Acetate
Control	25.5 ± 2.5	24.8 ± 1.7	30.4 ± 2.1	32.3 ± 1.8
0.25	32.2 ± 1.3	23.2 ± 2.2	25.9 ± 0.7*	30.7 ± 4.3
0.5	30.0 ± 2.4	24.7 ± 2.7	22.1 ± 1.3*	32.3 ± 2.9
1.0	33.9 ± 3.1*	21.1 ± 3.7	16.2 ± 1.0*	33.3 ± 4.1

Data represent the mean ± S.D. of three different experiments.
* Significantly different from control group ($P \leq 0.05$) using ANOVA followed by a Duncan multiple range test.

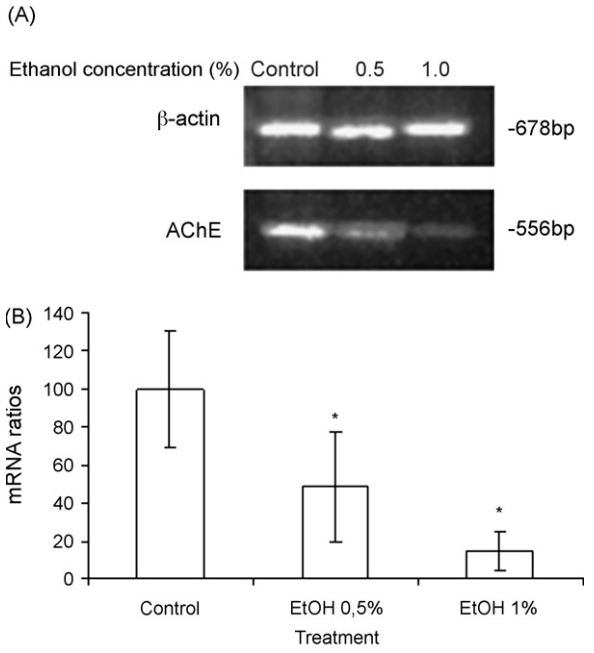

Fig. 1. AChE and β-actin mRNA expression in the brain of adult zebrafish. Fish were exposed to ethanol concentrations (0.5% and 1%). The brains RNA was isolated and RT-PCR products were subjected to electrophoresis on a 1% agarose gel (A). The enzyme/β-actin mRNA ratios were obtained by optical densitometry analysis (B). Three independent experiments were performed, with entirely consistent results. The data are expressed as means ± S.D. *Significantly different from control group ($P \leq 0.05$, ANOVA followed by Duncan post hoc).

The increase of ASCh hydrolysis promoted by ethanol exposure could be consequence of transcriptional control and/or post-translational regulation. RT-PCR analyses were performed when kinetic alterations were observed. Therefore, the ethanol concentrations tested were 0.5% and 1%. The concentration 0.5% of ethanol was also tested because a trend toward an increase of AChE activity was induced by this treatment. The relative amount of AChE transcripts were significantly reduced (52% and 86%) after 0.5% and 1% ethanol exposure, respectively (Fig. 1).

4. Discussion

In the present study, we have shown that ethanol can alter the activity and expression pattern of AChE in zebrafish brain. The relationship between cholinergic systems and operant tasks, exposure to novel stimuli, locomotor activity and the performance of spatial memory tasks is well established (Pepeu and Giovannini, 2004). The influence of ethanol has been described on these behavioral parameters in zebrafish (Gerlai et al., 2000).

Acute ethanol exposure enhanced the AChE activity, but when the same concentrations have been tested *in vitro*, AChE activity was not modified. There are several mechanisms that could regulate AChE after *in vivo* experiments, which include modifications at transcriptional or post-transcriptional levels. The neuronal responses to alcohol involve several hormone and neurotransmitter-activated signal transduction pathways, leading to short-term (acute) and long-term (chronic) changes in gene expression and neuronal function (Mailliard and Diamond, 2004).

In order to verify whether the AChE gene could be modulated when zebrafish were exposed to ethanol after 1 h, we have performed semi-quantitative RT-PCR experiments after 0.5% and 1% treatments. Interestingly, the results demonstrated that AChE mRNA levels were significantly decreased after 0.5% and 1% exposure, suggesting that the increase of AChE activity observed in this treatment is not directly related to a higher AChE gene expression. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. This signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors (Krishna et al., 2006). The phenomenon known as negative feedback loop (Salgado et al., 2001; Keseler et al., 2005), which is situated at the interface of genetic and metabolic networks, could explain the concomitant increase of ACh hydrolysis and the decrease of AChE mRNA levels in zebrafish brain after ethanol exposure. Ethanol could exert an influence on AChE post-translational modulation that in turn regulates its own expression. It was already demonstrated that chicken AChE activity in neurogenesis (Robitzki et al., 1997) and neural differentiation (Keller et al., 2001) is clearly regulated by a post-translational event. In this sense, zebrafish AChE sequence presents a high-predicted score of possible PKC phosphorylation sites (Thr271 and Thr761), according to analysis in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool. Therefore, the increase on AChE activity could be attributed to possible changes in phosphorylation state. In addition, it was also demonstrated that cellular expression of chicken AChE is regulated by the amount of the active AChE within neuronal tissues (Layer et al., 1992). Thus, zebrafish brain AChE activation induced by ethanol could be consequence of changes in phosphorylation state that down regulates its own expression.

Considering that ethanol metabolites could be involved in the observed effects, we performed *in vitro* assays testing acetaldehyde and acetate in order to ver-

ify the effects of ethanol metabolites on zebrafish brain AChE activity. Acetaldehyde, the first metabolite of ethanol catabolism pathway, inhibited AChE activity in a concentration-dependent manner. Studies have demonstrated the role of acetaldehyde in several neurochemical and pharmacological effects promoted by ethanol. It has been postulated that *in vivo* acetaldehyde production may play a role in the ethanol cytotoxicity, inducing neuronal degeneration (Takeuchi and Saito, 2005). It is not possible to exclude that an important mechanism for ethanol toxicity is lipid peroxidation through the induction of the formation of free radicals and/or acetaldehyde adducts, which could be associated with brain and others organs damage (Niemela, 2001; Sun and Sun, 2001; Nakamura et al., 2003).

Acetate, another product of this pathway, is a short-chain fatty acid that increases after ethanol administration and readily crosses the blood–brain-barrier, being metabolized in brain. This metabolite can be destined to acetyl-CoA, which is formed from acetate as well as can be metabolized for energy generation into CO_2 and water (Carmichael et al., 1991). Furthermore, evidence has suggested that extracellular acetate can be accumulated and released by cholinergic nerve terminals after stimuli (Carroll, 1997), resulting in the increase of ACh levels at synaptic cleft. Since acetate *in vitro* did not alter AChE activity, it is possible to exclude the involvement of this metabolite in the effects induced by ethanol.

Ethanol is able to modulate the action of several neurotransmitters and neuromodulators, including adenosine. This alcohol has been proposed to stimulate adenosine receptors by two mechanisms. The first involves metabolism of acetate, which requires ATP and yields AMP. The latter compound is converted into adenosine by the enzyme 5′-nucleotidase (Carmichael et al., 1991). The second mechanism involves an inhibition promoted by ethanol on type I equilibrative nucleoside transporter (ENT1), which leads to the accumulation of extracellular adenosine (Carroll, 1997). The stimulation of adenosine A_{2A} receptors leads to the activation of Gαs-coupled adenyl cyclase, increasing the generation of cAMP and acetylcholine release from rat hippocampus (Newton and Messing, 2006). Consequently, an increase of ACh levels promoted by adenosine A_{2A} receptors could also induce an enhancement of AChE activity by a stoichiometric effect, which could represent an important compensatory mechanism in order to maintain the control of ACh levels during ethanol exposure.

The search for biological alterations on cholinergic systems during alcohol exposure in zebrafish may not only render some important insights into the pathophysiology of alcohol dependence, but might also identify neurochemical and molecular mechanisms involved in the alcoholism.

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