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Ethanol and acetaldehyde alter NTPDase and 5′-nucleotidase from zebrafish brain membranes

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Abstract

Alcohol abuse is an acute health problem throughout the world and alcohol consumption is linked to the occurrence of several pathological conditions. Here we tested the acute effects of ethanol on NTPDases (nucleoside triphosphate diphosphohydrolases) and 5′-nucleotidase in zebrafish (*Danio rerio*) brain membranes. The results have shown a decrease on ATP (36.3 and 18.4%) and ADP (30 and 20%) hydrolysis after 0.5 and 1% (v/v) ethanol exposure during 60 min, respectively. In contrast, no changes on 5′-nucleotidase activity were observed in zebrafish brain membranes. Ethanol *in vitro* did not alter ATP and ADP hydrolysis, but AMP hydrolysis was inhibited at 0.5, and 1% (23 and 28%, respectively). Acetaldehyde *in vitro*, in the range 0.5–1%, inhibited ATP (40–85%) and ADP (28–65%) hydrolysis, whereas AMP hydrolysis was reduced (52, 58 and 64%) at 0.25, 0.5 and 1%, respectively. Acetate *in vitro* did not alter these enzyme activities. Semi-quantitative expression analysis of NTPDase and 5′-nucleotidase were performed. Ethanol treatment reduced NTPDase1 and three isoforms of NTPDase2 mRNA levels. These findings demonstrate that acute ethanol intoxication may influence the enzyme pathway involved in the degradation of ATP to adenosine, which could affect the responses mediated by adenine nucleotides and nucleosides in zebrafish central nervous system.
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1. Introduction

Alcohol abuse is a significant public health problem. Its consumption affects wide proportion of the general population, being responsible for the occurrence of several mental disorders throughout the world. The acute effects are characterized by a variety of behavioral changes related to motor coordination, sensory perception and cognition (Fleming et al., 2001). Ethanol promotes several biochemical and physiological alterations on central nervous system, involving specific neurotransmitter systems and intricate signaling pathways (Chandler et al., 1997; Esel, 2006).

The zebrafish, *Danio rerio*, is a small freshwater teleost emerging as an important model in genetic and developmental neurobiology (Zon and Peterson, 2005). Zebrafish genes present a high degree of conservation and share a 70–80% homology to those of humans (Dooley and Zon, 2000).

Therefore, zebrafish has become an excellent model system for identifying and understanding the genes responsible for normal vertebrate development, as well genes that regulate sensitivity and resistance to toxicants, including ethanol.

Recently, the teratogenic properties of ethanol have been established in zebrafish, through developmental abnormalities as fetal alcohol syndrome (FAS) and induction of cell death in the CNS (Dlugos and Rabin, 2003). In addition, ethanol influences behavioral parameters, such as locomotor activity, aggression, group preference and pigment response in this specie (Gerlai et al., 2000; Reimers et al., 2004). The gene of alcohol dehydrogenase (ADH), the primary enzyme responsible for the degradation of alcohol, has been described and cloned in this specie (Reimers et al., 2004a; Dasmahapatra et al., 2001).

Extracellular ATP can play an important role in synaptic transmission, acting as a neurotransmitter and/or a neuromodulator (Cunha and Ribeiro, 2000; Burnstock, 2004). In the literature, both ionotropic P2X and G protein-coupled P2Y receptors have already been characterized in this specie (Kucenas et al., 2003). Signaling events induced by extracellular adenine nucleotides are controlled by the action of a variety of surface-located enzymes known as nucleotidases (Zimmermann, 2001; Robson et al., 2006). There are important mechanisms involved in the control of ligand concentrations and hence regulate the activation of purinoceptors. Nucleotidases constitute a highly refined system for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and nucleoside formation. The hydrolysis of ATP to AMP is catalyzed by the families of nucleotidases named nucleoside triphosphate diphosphohydrolase (NTPDases) and ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP). NTPDase1 was already cloned and characterized on Torpedo electric organ (Martin-Satue et al., 2007). Moreover, the E-NPP family is involved in the cleavage of extracellular diadenosine polyphosphates in brain (Asensio et al., 2007). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of a $5'$-nucleotidase (Zimmermann, 1992). 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).

Recently, we characterized the presence of NTPDase and $5'$-nucleotidase activities in brain membranes of zebrafish (Rico et al., 2003). Orthologous NTPDase1, three NTPDases2 and $5'$-nucleotidase genes were identified in zebrafish genome, presenting expression in brain (Rico et al., 2006). Thus, there are important mechanisms involved in the control of nucleotide and nucleoside concentrations, regulating the purinergic neurotransmission.

In order to understand the control of nucleotide levels in zebrafish brain and considering that: (i) ethanol mediates its actions through several excitatory or inhibitory neurotransmitter systems; (ii) P2X receptors play a role in mediating cellular and behavioral effects of ethanol (Franke and Illes, 2006); (iii) ethanol activates signal transduction pathways, leading to changes in gene expression and neuronal function (Diamond and McIntire, 2002), the present study evaluated in vivo effects of ethanol on activities and the pattern of expression of the nucleotidases. In addition, in vitro effects of ethanol and its metabolites, acetaldehyde and acetate, were also investigated in zebrafish brain membranes.

2. Material and methods

2.1. Animals

Adult zebrafish of both sexes were obtained from commercial supplier and acclimated for at least 2 weeks in a 50 L aquarium, with feeding done twice daily. The fish were kept between $25 \pm 2\,^\circ C$ under a natural light–dark photoperiod. The use of animals was according to the National Institute of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering to the animals, as well the number used. The Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under the number 477/05—CEP.

2.2. Chemicals

Ethanol (C_2H_6O) and acetate ($C_2H_4O_2$) were purchased from Merck, and acetaldehyde (C_2H_4O) from Fluka. Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.3. In vitro assays

Ethanol, acetaldehyde and acetate were added to reaction medium before the preincubation with the enzyme and maintained throughout the enzyme assays. The compounds were tested in the following final concentrations: 0.25, 0.5, and 1% in volume.

2.4. Acute ethanol exposure

For the in vivo treatments, animals were introduced to the test aquariums (20 L) containing solutions of ethanol at three different concentrations (0.25, 0.5, and 1% in volume). The animals were maintained in the test aquarium during 1 h and, immediately after the exposure, the fish were euthanized and the membrane preparations were performed. The acute ethanol exposure has been performed as described previously (Gerlai et al., 2000), which was able to promote significant changes in locomotor activity of zebrafish. The alcohol treatment at 0.25 and 0.5% led to a significant increase of locomotor activity, when compared to control and 1% alcohol treatment.

2.5. Membrane preparation

The preparation of brain membranes was performed as described previously (Barnes et al., 1993). Zebrafish were euthanized, their brains were removed of cranial skull by dissection technique and briefly homogenized in 60 volumes (v/w) of chilled 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 samples were centrifuged at $1000 \times g$ during 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at $40,000 \times g$. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at $40,000 \times g$. This fresh-thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at $2–4\,^\circ C$ throughout preparation.

2.6. Enzyme assays

The conditions of NTPDase and $5'$-nucleotidase assay were performed as described previously (Rico et al., 2003; Senger et al., 2006). Zebrafish brain membranes (3–10 μg protein) 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) 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 (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped by the addition of trichloroacetic acid in a final concentration of 5% and the samples were chilled on ice for 10 min. Samples were then removed and it was added 1 ml of a mixture containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% Malachite Green in order to determinate the inorganic phosphate released (Pi) (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nanomol of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

2.7. Protein determination

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

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

In order to obtain NTPDase1 and NTPDase2 zebrafish orthologous genes, the mouse proteins sequences (AAH11278 and NP_033979) were used as query. NCBI Blast searches of GenBank yielded one zebrafish sequence similar to NTPDase1 and three different isoforms of NTPDase2. PCR NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv primers were designed based on sequences obtained in GenBank and the optimal conditions for primers annealing were determined (Table 1). The β-actin primers were designed 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. PCR reactions for different NTPDase2 and β-actin genes were performed in a total volume of 20 μl, 0.1 μM primers (Table 1), 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). The PCR conditions for NTPDase1 were similar to those described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min for annealing temperature (see Table 1), 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, a negative control was included. PCR products were analyzed on 1.5% agarose gel, containing ethydium bromide and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as molecular marker and normalization was performed employing β-actin as a constitutive gene.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means ± S.D. A Duncan multiple test range as post-hoc was performed, considering a level of significance of 5%.

3. Results

Nucleotidase activities were evaluated after acute ethanol treatment in zebrafish brain membranes. After 1 hour of ethanol exposure at varying concentrations at the range of 0.25 to 1%, this alcohol was able to inhibit NTPDase activity. There was a significant decrease on ATP (36.3 and 18.4%) and ADP hydrolysis (30 and 20%) at 0.5 and 1%, respectively (Fig. 1A and B). 5′-Nucleotidase was not altered in the concentrations tested (data not shown).

Ethanol *in vitro* did not promote a significant effect on ATP and ADP hydrolysis (data not shown), while the AMP hydrolysis was reduced (23.3 and 28.1%) at 0.5 and 1%, respectively (Fig. 2). Therefore, our results have shown that ethanol was able to inhibit ATP and ADP hydrolysis *in vivo*, but not *in vitro*. These findings lead us to the investigation of possible indirect effects of ethanol, which could be mediated by its metabolites. Thus, we tested the *in vitro* effect of the metabolites, acetaldehyde and acetate, produced through ethanol degradation pathway (Table 2). Acetaldehyde, the first product of ethanol catabolism, induced a significant effect on nucleotidases, decreasing ATP hydrolysis in a dose-dependent manner (40.5, 65.8 and 85.5%) at the concentrations 0.25, 0.5, and 1%, respectively. When acetaldehyde was added to the enzyme assay, ADP hydrolysis also presented an inhibitory effect (28.5, 44.7, and 64.7%) at the concentrations 0.25, 0.5, and 1%, respectively. There was a significant decrease of AMP hydrolysis in all concentrations of acetaldehyde tested (52.2, 58.7 and 64.4% at the concentrations 0.25, 0.5 and 1%, respectively). In the next step of ethanol catabolism, acetaldehyde is metabolized to acetate. Acetate *in vitro* did not induce alterations on NTPDase and 5′-nucleotidase activities in the concentrations tested (Table 2).

The inhibitory effect promoted by ethanol could be a consequence of transcriptional control. The semi-quantitative

Table 1
PCR primer design

Enzymes	Sequences (5′-3′)	Annealing temperature (°C)	PCR product (bp)	GenBank accession number
NTPDase1	CCCATGGCACAGGCCGGTTG (forward) GCAGTCTCATGCCAGCCGTG (reverse)	54	380	AAH78240
NTPDase2_mg[*]	GGAAGTGTTTGACTCGCCTTGCACG (forward) CAGGACACAAGCCCTTCCGGATC (reverse)	64	554	XP_697600
NTPDase2_mq[*]	CCAGCGGATTTAGAGCACGCTG (forward) GAAGAACGGCGGCACGCCAC (reverse)	64	313	XP_687722
NTPDase2_mv[*]	GCTCATTTAGAGGACGCTGCTCGTG (forward) GCAACGTTTTCGGCAGGCAGC (reverse)	64	263	AAH78419
β-Actin	GTCCCTGTACGCCTCTGGTCG (forward) GCCGGACTCATCGTACTCCTG (reverse)	54	678	AAC13314

[*] Correspond to the two first amino acids residues of the protein sequence.
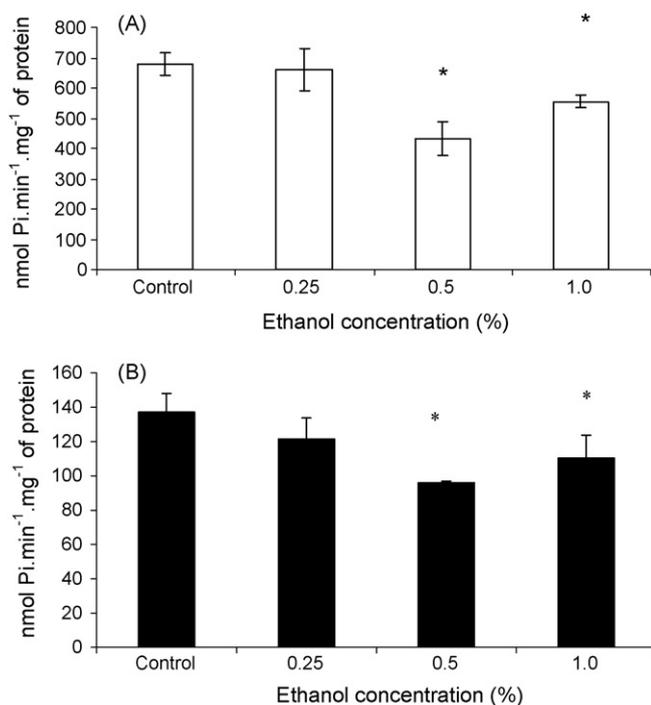
Fig. 1. Effect of acute ethanol treatment on nucleotidase activities in zebrafish brain. The ATP (A), ADP (B) hydrolysis were evaluated in three different concentrations (0.25, 0.5 and 1.0%). Data are expressed as mean ± S.D. of at least three different experiments. Asterisk (*) indicates significant difference from control group ($P \leq 0.05$).

RT-PCR analyses were performed when kinetic alterations had occurred. For this reason, 5′-nucleotidase and the concentrations of 0.25% ethanol for NTPDase1 and NTPDases2 isoforms were not analyzed. Ethanol exposure decreased the expression of NTPDases in zebrafish brain (Fig. 3). NTPDase1, NTPDase2_mg and NTPDase2_mv presented a decrease in the level of transcripts after exposure to ethanol 0.5%, while that NTPDase2_mq transcription apparently was not affected. Interestingly, all NTPDases demonstrated a reduction in the transcript levels at 1% of ethanol treatment.
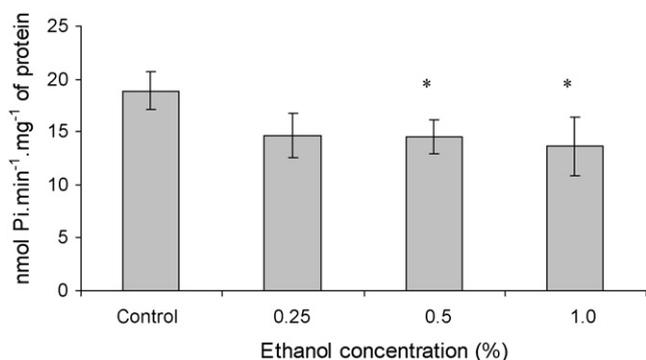

Fig. 2. In vitro effect of varying concentrations of ethanol on AMP hydrolysis in zebrafish brain membranes. Bars represent the means ± S.D. of at least three different experiments. Asterisk (*) indicates significant different from control group ($P \leq 0.05$).

4. Discussion

The results presented demonstrate the influence of ethanol on nucleotidase activities and expression patterns in zebrafish brain. Acute treatment significantly inhibited NTPDase activity at higher concentrations tested. However, 5′-nucleotidase did not present any significant change after in vivo exposure.

To verify if the inhibition observed in ATP and ADP hydrolysis from zebrafish brain could be due to alteration on gene expression, NTPDase and 5′-nucleotidase primers were synthesized and RT-PCR experiments were conducted. The results have shown a decrease on NTPDase1, NTPDase2_mg, and NTPDase2_mv transcript levels after ethanol treatment.

Since these enzymes contribute to maintenance of physiological effects of extracellular ATP, ADP, AMP and adenosine, the influence of the enzymatic cascade involved in the control of these nucleotides and nucleosides have been proposed in several pathophysiological situations (Agteresch et al., 1999). Our results suggest that the inhibitory effect on ATP and ADP hydrolysis observed after ethanol exposure could induce an increase in the extracellular ATP levels and a consequent decrease of adenosine levels. Previous studies from our laboratory have shown a significant decrease on ATP (26 and 45%) and ADP hydrolysis (26 and 30%) at 0.5 and 1% methanol exposure, respectively, suggesting that the alcohols could present a similar effect on these enzyme activities.

Considering that ATP is an important excitatory neurotransmitter in CNS (Di Iorio et al., 1998), the inhibition of ATP hydrolysis could promote several processes related to brain excitability. As the massive release of extracellular ATP leads to excitotoxicity and cell death via activation of $P2X_7$ receptor in neuronal cells, studies have related this nucleotide to neuropathological events targeting the CNS (Le Feuvre et al., 2002).

In order to verify the direct effect of ethanol on nucleotidase activities, in vitro assays were performed in zebrafish brain membranes. The NTPDase was not altered, but 5′-nucleotidase activity was inhibited at 0.25, 0.5, and 1%. Similar effects on NTPDase activities were observed after in vitro methanol exposure whereas 5′-nucleotidase was not altered by this alcohol (Rico et al., 2006).

Alcohol is believed to interact with biological membranes due to its lipophilic nature. The direct interaction of the ethanol molecule, per se, with membrane structures is complex and may involve such membrane components (Lovinger et al., 1989; Carrasco et al., 2007). Vieira et al. (2004) have shown that NTPDase-like activity, after exposure in vitro, is sensitive to several inhibitors in membranes from liver and kidney of fish, chicken and rats. However, ethanol treatment was able to inhibit the NTPDase activity while alterations were not observed after in vitro assays. These results permit to conclude that ethanol could not act directly, but indirectly through its metabolites, acetaldehyde and acetate. Generation of oxygen free radicals and reactive aldehydes as a result of excessive ethanol consumption has been well established and have indicated that acetaldehyde, and the aldehydic products of lipid peroxidation can bind to proteins in tissues forming stable

Table 2
In vitro effects of acetaldehyde and acetate on ATP, ADP and AMP hydrolysis in zebrafish brain membranes

	Acetaldehyde			Acetate		
	ATP	ADP	AMP	ATP	ADP	AMP
Control	613.1 ± 66.0	123.5 ± 16.1	21.3 ± 2.6	630.1 ± 125.2	114.3 ± 6.6	22.2 ± 2.3
0.25	$365.2 \pm 71.1^{*}$	$88.3 \pm 2.5^{*}$	$10.2 \pm 1.7^{*}$	598.7 ± 115.1	128.8 ± 20.6	22.4 ± 4.9
0.5	$210.2 \pm 57.6^{*}$	$68.4 \pm 8.8^{*}$	$8.7 \pm 2.5^{*}$	586.8 ± 144.3	124.2 ± 19.4	19.3 ± 3.9
1.0	$89.5 \pm 22.3^{*}$	$43.7 \pm 6.1^{*}$	$7.6 \pm 3.2^{*}$	553.1 ± 116.6	103.9 ± 4.8	18.4 ± 3.5

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

adducts (Niemela, 2001). Moreover, neuronal responses to alcohol involve several hormone- and neurotransmitter-activated pathways, leading short-term (acute) and long-term (chronic) changes in gene expression and neuronal function (Diamond and McIntire, 2002; Garige et al., 2006). Thus, the *in vivo* inhibitory effect on transcriptional and kinetic parameters could be associated to toxicity formation of adducts and oxidation stress.

After alcohol consumption, ethanol is metabolized in the liver through several mechanisms (Ramchandani et al., 2001), including alcohol dehydrogenase (ADH), cytocrome P4502E1 (CYP2E1) and catalase. Acetaldehyde hydrolysis is mainly mediated by aldehyde dehydrogenase (ALDH), producing acetate. It is already known that there are two oxidative pathways for metabolizing ethanol to acetaldehyde in brain: catalase may be responsible for about 60% of the process (Zimatkin et al., 2006) while cytocrome P450 (CYP2E1) is involved in the metabolic conversion of ethanol to reactive oxygen species (Sun and Sun, 2001; Yadav et al., 2006). Furthermore, it is consolidated that acetaldehyde and acetate play a key role in the brain mediating some actions of ethanol (Israel et al., 1994; Deitrich, 2004). It is well established that acetaldehyde mediates the toxic effects of ethanol, and studies were aimed at unraveling its effects in pathological conditions (Quertemont et al., 2005). In order to understand the possible effect of products of ethanol metabolism, acetaldehyde and acetate were tested *in vitro* on nucleotidase activities.

Fig. 3. Gene expression patterns after acute ethanol exposure. The figure shows β-actin, NTPDase1, NTPDase2_mg, NTPDase2_mq and NTPDase2_mv mRNA expression in the brain of adult zebrafish. Fish were exposed to ethanol concentrations (0.25, 0.5 and 1.0%), the brains were excised and total RNA was isolated being subjected to RT-PCR for the indicated targets. RT-PCR products were subjected to electrophoresis on a 1.5% agarose gel. Three independent experiments were performed, with entirely consistent results.

Acetaldehyde promoted an inhibition on NTPDase activities in a dose-dependent manner ranging from 0.25 to 1%, while the activity of 5′-nucleotidase was equally inhibited at all concentrations tested.

Acetate is a molecule that promotes significant effects on CNS that can either potentiate or antagonize the effects of ethanol molecule (Carmichael et al., 1991). Acetate *in vitro* was not able to modify nucleotidase activities. Ethanol has been proposed to stimulate adenosine receptors by two mechanisms. The first involves metabolism of ethanol by liver, which generates acetate, which is converted to Acetyl-CoA, a process that requires ATP and yields AMP (Carmichael et al., 1991). This AMP is converted to adenosine by the 5′-nucleotidase (Bianchi and Spychala, 2003), leading to an increase of adenosine levels. The second mechanism has been demonstrated through the inhibition of the type I equilibrative nucleoside transporter (ENT 1), which leads to accumulation of extracellular adenosine (Choi et al., 2004). The association of NTPDase and 5′-nucleotidase can promote the hydrolysis of ATP, ADP and AMP, leading to the formation of adenosine. To prevent adenosine accumulation, the inhibitory responses promoted by ethanol on NTPDases could be a compensatory mechanism to avoid a significant increase of adenosine levels, which can lead to desensitization of the adenosine receptors (Kiselevski et al., 2003). The action of ethanol on neuromodulatory function of adenosinergic system regulates the release of several neurotransmitters (Fredholm et al., 2005).

Recent evidence indicates that ethanol modulates the function of specific intracellular signaling cascades, including those that contain cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent, protein kinase A (PKA) and protein kinase C (PKC) (Newton and Messing, 2006). Zebrafish NTPDase1 and all NTPDases2 isoforms protein sequences present possible PKC phosphorylation sites, according to analysis performed in NetPhosK, a kinase-specific prediction of protein phosphorylation site tool. Furthermore, PKC phosphorylates numerous proteins, including transcription factors, which regulate the activity of many genes in the cell nucleus (Dohrman et al., 1997). Besides the decrease in NTPDase transcript levels, the inhibition on these enzyme activities could also be attributed to ethanol effect on signaling pathways involved in the possible post-translational modulation of these enzymes.

In summary, these findings demonstrate the actions induced by ethanol and its metabolites on nucleotidases in zebrafish brain. This investigation evaluated the relationship between ethanol, recognized for acting in neurotransmission, and the
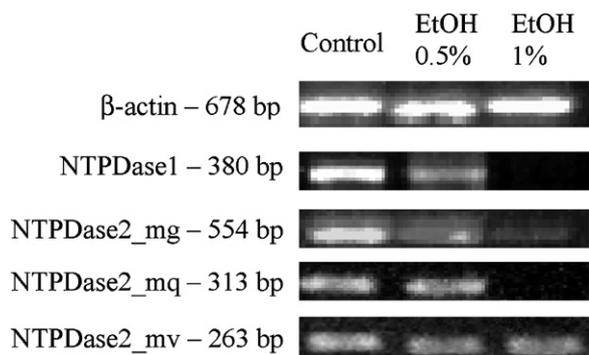
enzymes responsible for the hydrolysis of the neurotransmitter ATP to adenosine. The changes induced by acute ethanol treatment on nucleotidases suggest that the purinergic system is an interesting target for potential pharmacological studies. Our results could help to clarify the importance of neurochemical effects on purine metabolism associated to alcohol consumption.

Acknowledgements

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