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Exposure to Hg^{2+} and Pb^{2+} changes NTPDase and ecto-5′-nucleotidase activities in central nervous system of zebrafish (*Danio rerio*)

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Abstract

Neurotransmission can be affected by exposure to heavy metals, such as mercury and lead. ATP is a signaling molecule that can be metabolized by a group of enzymes called ecto-nucleotidases. Here we investigated the effects of mercury chloride ($HgCl_2$) and lead acetate ($Pb(CH_3COO)_2$) on NTPDase (nucleoside triphosphate diphosphohydrolase) and ecto-5′-nucleotidase activities in zebrafish brain membranes. In vitro exposure to $HgCl_2$ decreased ATP and ADP hydrolysis in an uncompetitive mechanism and AMP hydrolysis in a non-competitive manner. $Pb(CH_3COO)_2$ inhibited ATP hydrolysis in an uncompetitive manner, but not ADP and AMP hydrolysis. In vivo exposure of zebrafish to $HgCl_2$ or $Pb(CH_3COO)_2$ (20 μg/L, during 24, 96 h and 30 days) caused differential effects on nucleotide hydrolysis. $HgCl_2$, during 96 h, inhibited the hydrolysis of ATP, ADP and AMP. After 30 days of exposure to $HgCl_2$, ATP hydrolysis returned to the control levels, ADP hydrolysis was strongly increased and AMP hydrolysis remained inhibited. Exposure to $Pb(CH_3COO)_2$ during 96 h caused a significant decrease only on ATP hydrolysis. After 30 days, $Pb(CH_3COO)_2$ promoted the inhibition of ATP, ADP and AMP hydrolysis. Semi-quantitative RT-PCR analysis showed no changes in the expression of NTPDase1 and 5′-nucleotidase, following 30 days of exposure to both metals. This study demonstrated that Hg^{2+} and Pb^{2+} affect the ecto-nucleotidase activities, an important enzymatic pathway for the control of purinergic signaling.

Keywords: NTPDase; Ecto-5′-nucleotidase; Heavy metals; Mercury; Lead; Zebrafish

1. Introduction

Heavy metals, such as mercury and lead are important environmental contaminants, which can reach aquatic systems derived from effluents of industrial, urban and

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mining sources. These substances present severe risk to the aquatic biota and humans, even at sublethal concentrations (Baatrup, 1991; Jarup, 2003). Animals exposed to Hg^{2+} and Pb^{2+} have adverse developmental, reproductive, neurological and behavioral effects. Many cellular processes are affected by exposure to Hg^{2+} and Pb^{2+} and the correct function of central nervous system can be impaired by neurochemical changes (Aguilar and Kostrzewa, 2004). Synaptic transmission can be altered after exposure to these heavy metals. Changes in the release, extracellular metabolism and/or uptake and expression of components of neurotransmitter systems have been related to toxic effects observed in heavy metals-exposed animals (Cooper and Manalis, 1983).

ATP is a primitive signaling molecule that has been retained as a cotransmitter in every nerve type in both peripheral and central nervous system (Burnstock, 2004). This molecule is released to the synaptic cleft in a calcium-dependent manner, where it can act as a fast neurotransmitter or as a modulator, regulating the activity of other transmitter substances (Cunha and Ribeiro, 2000). ATP exerts its effects through purinoceptors, divided in two major classes, ionotropic P2X and metabotropic P2Y receptors (Ralevic and Burnstock, 1998). At the synapse, ATP can be metabolized by a group of enzymes called ecto-nucleotidases, which includes NTPDase family (nucleoside triphosphate diphosphohydrolase) and ecto-5′-nucleotidase. The final product of this enzyme cascade is the nucleoside adenosine, an important neuromodulator that acts on G-protein-coupled receptors, named A_1, A_{2A}, A_{2B} and A_3 (Ribeiro et al., 2003).

Ecto-nucleotidases are ubiquitous enzymes with a broad phylogenetic distribution, occurring in many vertebrate tissues. NTPDases present the ability to hydrolyze triphosphate and diphosphate nucleotides. Mammalian NTPDases1–3 and 8 are extracellular enzymes that can be classified according the ATP/ADP preference (Zimmermann, 2001; Bigonesse et al., 2004). NTPDase1 (CD39) hydrolyzes ATP and ADP almost equally well. NTPDase2 (CD39L1) has a large preference for ATP over ADP. NTPDase3 (CD39L3 or HB6) and NTPDase8 slightly prefer ATP over ADP by a ratio of about 3 and 2, respectively. The nucleotide AMP, which is the final product of ATP and ADP hydrolysis promoted by NTPDases, can be hydrolyzed by the action of an ecto-5′-nucleotidase, producing the neuromodulator adenosine. Ecto-5′-nucleotidase has a pivotal role together with the NTPDases in regulating the concentration of extracellular nucleotides and nucleosides to the purinoceptors (Zimmermann, 2001).

There are few studies demonstrating the effect of heavy metals on ecto-nucleotidases. Oliveira et al. (1994) investigated the in vitro and in vivo effect of $HgCl_2$ on synaptosomal ATP diphosphohydrolase from cerebral cortex of developing rats. These authors observed contrasting results, whereas ATP and ADP hydrolysis were inhibited in vitro, exposure in vivo did not affect the nucleotide hydrolysis. Furthermore, Moretto et al. (2004) verified the subchronic (0.1 mg/kg; 30 doses/30 days) effect of $HgCl_2$ on NTPDase and 5′-nucleotidase activity of adult rats, showing a significant increase on NTPDase activity, but not on 5′-nucleotidase activity.

Zebrafish is a consolidated model system in neuroscience and toxicological studies (Linney et al., 2004; Senger et al., 2005). The zebrafish genome project has demonstrated regions of syntenic relationship with human genome (Barbazuk et al., 2000). Purinoceptors were already identified in this teleost (Kucenas et al., 2003) and we characterized the presence of a NTPDase and an ecto-5′-nucleotidase activities in brain membranes of zebrafish (Rico et al., 2003; Senger et al., 2004). These enzymes were cation-dependent, with a maximal rate for nucleotide hydrolysis in a pH range of 7.5–8.0 in the presence of Ca^{2+} for NTPDase and Mg^{2+} for ecto-5′-nucleotidase (Rico et al., 2003; Senger et al., 2004).

Considering that mercury and lead are important environmental contaminants and previous studies have demonstrated the presence of purinergic receptors and enzyme activities involved in extracellular catabolism of nucleotides in zebrafish brain, the aim of present study was to investigate the effect of mercury chloride and lead acetate on NTPDase and ecto-5′-nucleotidase activities and expression in central nervous system of zebrafish.

2. Materials and methods

2.1. Animals

Adult zebrafish were obtained from commercial suppliers and maintained at least for 2 weeks in a 50-L aquarium before the experiments. The fish of both sexes were kept at $25 \pm 2\,^{\circ}C$ under a natural light–dark photoperiod. Animals feeding and maintenance of fishes were done according to Westerfield (2000). All procedures for the use of animals were according to the National Institute of Health Guide for Care and Use of Laboratory.

2.2. Chemicals

Mercury chloride ($HgCl_2$, CAS Number 7487-94-7) and lead acetate [$Pb(CH_3COO)_2$, CAS Number 301-04-2] were purchased from Quimibrás Indústrias Químicas (Brazil).

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. Treatments

The concentrations used for in vitro experiments were chosen according to previous studies evaluating the effect of mercury on nucleotidase activities (Oliveira et al., 1994). For the in vitro assays, mercury chloride or lead acetate at the final concentrations of 0.05–1 mM were added to reaction medium, pre-incubated for 10 min with the brain membranes and maintained throughout the enzyme assay.

For the in vivo treatments, animals were introduced to the test aquarium (20 L) containing solutions of mercury or lead at the final concentration of 20 μg/L, which has been reported in the aquatic environment (Berzas Nevado et al., 2003; Jha et al., 2003). The animals were maintained in the test aquarium for different exposure periods: 24, 96 h or 30 days.

2.4. Membrane preparation

Brain membranes were prepared according to Barnes et al. (1993). Whole zebrafish brains were 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 glass-Teflon homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at 40,000 × *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and recentrifuged for 20 min at 40,000 × *g*. This freeze-thaw-wash procedure was used to ensure lysis of the membranes. The final pellet was resuspended and used in the enzyme assays. The material was maintained at 2–4 °C throughout preparation.

2.5. Enzyme assays

Brain membranes of zebrafish (3–5 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 ecto-5′-nucleotidase activity) in a final volume of 200 μL (Rico et al., 2003; Senger et al., 2004). The samples were pre-incubated for 10 min at 37 °C. The reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM and stopped by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) (Chan et al., 1986). Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released min^{-1} mg of $protein^{-1}$. All enzyme assays were run at least in triplicate.

2.6. Protein determination

Protein was measured by the Coomassie blue method, using bovine serum albumin as a standard (Bradford, 1976).

2.7. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To obtain NTPDase1 and 5′-nucleotidase zebrafish orthologous genes, the mouse proteins sequences (AAH11278 and AAC13542) were used. When mouse sequences were used as query, NCBI Blast searches of GenBank yielded one zebrafish sequence similar to NTPDase1 (AAH78240) and another (NP_957226) assigned as 5′-nucleotidase. From these sequences, specific zebrafish primers of NTPDase1 (DrNTPDase1F 5′-CCC ATG CGA CAG GCC GGT T-3′ and DrNTPDase1R 5′-GCA GTC TCA TGC CAG CCG TG-3′) and 5′-nucleotidase (DrCD73F 5′-ACC TCC GAG GAG TGT CGC TTT CG-3′ and DrCD73R 5′-CCT TGT TGG GGA CCA GCG GTT C-3′) were designed. The β-actin zebrafish primers (forward: 5′-GTC CCT GTA CGC CTC TGG TCG-3′ and reverse: 5′-GCC GGA CTC ATC GTA CTC CTG-3′) were 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 5′-nucleotidase and β-actin genes were performed in a total volume of 20 μL using 1 μL of RT reaction mix, 0.1 μM of each primer (indicated below), 0.2 μM of each dNTP, 2 mM of $MgCl_2$ and 0.5 U of Taq DNA polymerase (Invitrogen). The PCR conditions for NTPDase1 were similar as described above, except that 1.5 mM of $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, negative control was included. Six microliters of the PCR product were analyzed on a 1.5% agarose gel.

2.8. Statistical analysis

Data were expressed as means ± S.D. and analyzed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant.

3. Results

The in vitro effects of mercury chloride and lead acetate on NTPDase and 5′-nucleotidase activities were evaluated in brain membranes of zebrafish. There was a significant decrease in ATP hydrolysis in all

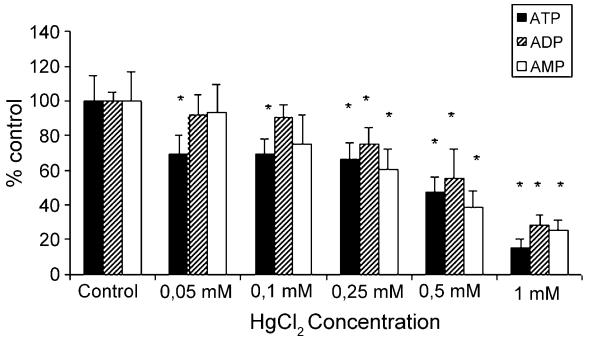

Fig. 1. In vitro effect of varying concentrations of Hg^{2+} on ATP, ADP and AMP hydrolysis in zebrafish brain membranes. Bars represent mean ± S.D. of four independent experiments. The control specific activities for ATP, ADP and AMP hydrolysis were 567.5 ± 83.8, 161.1 ± 8.8 and 23.4 ± 3.9 nmol Pi min^{-1} mg^{-1} protein, respectively. *Significantly different from control group (without metal added) ($P < 0.05$).

concentrations of mercury chloride tested (at the range 0.05–1 mM) and the inhibitory effect varied from 31 to 85%, respectively (Fig. 1). Mercury chloride also had an inhibitory effect in the concentrations of 0.25–1 mM for ADP (25–72%) and AMP hydrolysis (30–63%), respectively (Fig. 1). Lead acetate significantly inhibited only ATP hydrolysis in brain membranes of zebrafish at the concentrations of 0.25–1 mM, but not altered ADP and AMP hydrolysis (Fig. 2).

To verify if mercury and lead can bind to ecto-nucleotidases, we performed an in vitro experiment with DTT (Fig. 3). The nucleotide hydrolysis were measured in the absence (control group) or in the presence of the metals (Hg^{2+} (Fig. 3A) or Pb^{2+} (Fig. 3B) at 0.5 mM); pre-incubated 5 min with DTT (1 mM) and after 5 min with the membrane preparation (E) (group DTT + E); or

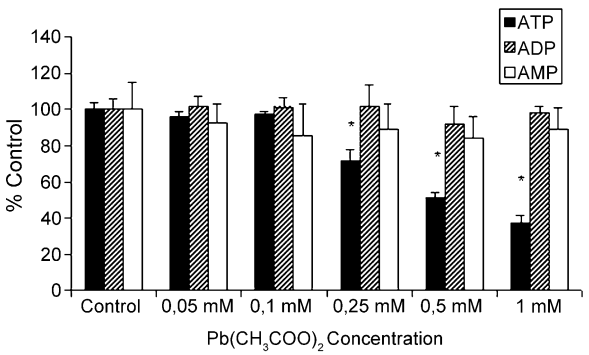

Fig. 2. In vitro effect of varying concentrations of Pb^{2+} on ATP, ADP and AMP hydrolysis in zebrafish brain membranes. Bars indicate mean ± S.D. of four independent experiments. Control specific activities of ATP, ADP and AMP hydrolysis were 605.8 ± 28.4, 141 ± 7.7 and 19.9 ± 3.2 nmol Pi min^{-1} mg^{-1} protein, respectively. *Significantly different from control (without metal added) ($P < 0.05$).

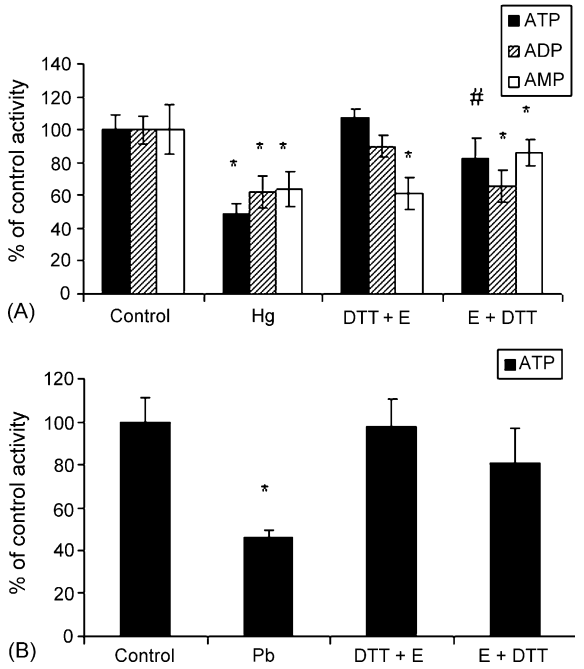

Fig. 3. Effect in vitro of DTT on the inhibition promoted by Hg^{2+} (A) or Pb^{2+} (B) on ecto-nucleotidase activities in zebrafish brain membranes. The nucleotide hydrolysis were measured in the absence (control group) or in the presence of the metals (Hg^{2+} or Pb^{2+} at 0.5 mM); pre-incubated 5 min with DTT (1 mM) and after 5 min with the enzyme (DTT + E); or pre-incubated 5 min with the enzyme and after more 5 min with DTT (1 mM) (E + DTT). *Significantly different from control (without metal added) ($P < 0.05$). #Statistical difference in relation to DTT + E group.

pre-incubated 5 min with the membrane preparation and after more 5 min with DTT (1 mM) (group E + DTT). Our experiments demonstrated that DTT, when added before the enzyme in the reaction medium (DTT + E), can revert the inhibitory effect promoted by both heavy metals. The addition of DTT after the pre-incubation of the metals with the enzyme (E + DTT), partially recovered only the inhibition promoted by $Hg^{2+}$and totally reverted the inhibition promoted by Pb^{2+} in ATP hydrolysis.

The interaction kinetics of these metals with NTPDase or 5′-nucleotidase in brain membranes of zebrafish were determined. The Lineweaver–Burk double reciprocal plot was analyzed over a range of substrates (0.1–0.25 mM) in the absence and in the presence of the mercury chloride (Fig. 4) or lead acetate (Fig. 5). The data indicated that mercury chloride inhibited ATP and ADP hydrolysis in an uncompetitive manner (Fig. 4A and B, respectively). The inhibition promoted by mercury chloride in the AMP hydrolysis appeared to operate by a non-competitive mechanism; however, this interpretation is based on a small number of data points (Fig. 4C).

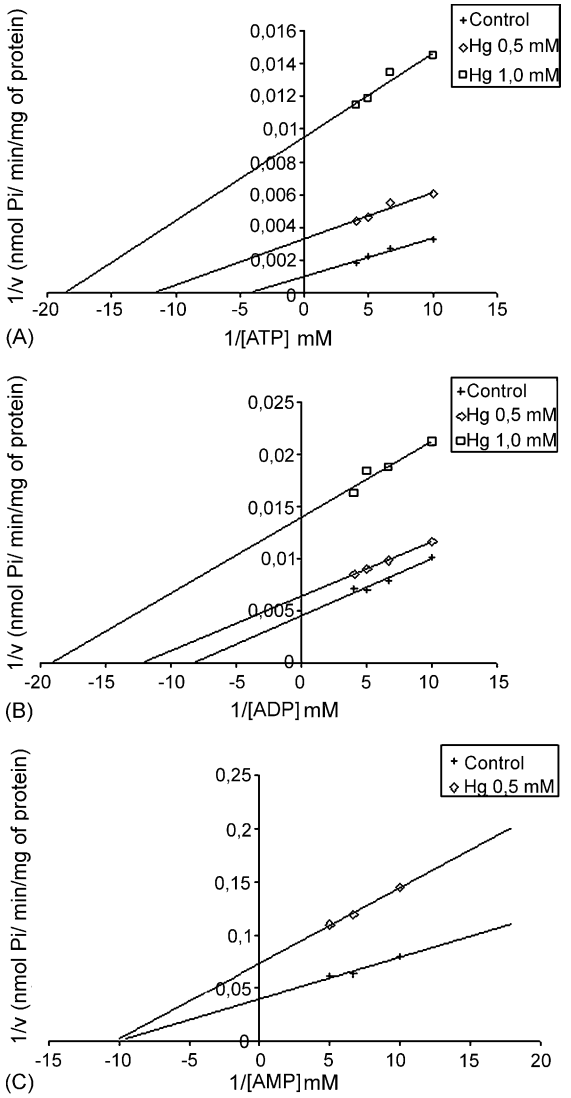

Fig. 4. Lineweaver–Burk plot of ATP (A), ADP (B) and AMP (C) hydrolysis and their interaction with Hg^{2+}. The concentration of the nucleotides varied from 0.1 to 0.250 mM in absence (+) and in the presence of 0.5 mM (◇) and 1 mM (□) of Hg^{2+}. All experiments were repeated at six to eight times and similar results were obtained. The data showed represent a typical experiment.

In relation to the inhibitory effect promoted by lead acetate, the data showed an uncompetitive inhibition for ATP hydrolysis (Fig. 5).

To evaluate the in vivo effect of these heavy metals in ecto-nucleotidases, animals were exposed to a treatment with mercury chloride and lead acetate, at a concentration of 20 μg/L during 24, 96 h and 30 days. There was no observed mortality in the treated and control groups in the different exposure times. Furthermore, no changes in the swimming pattern were observed in metal-treated fish when compared to control group.

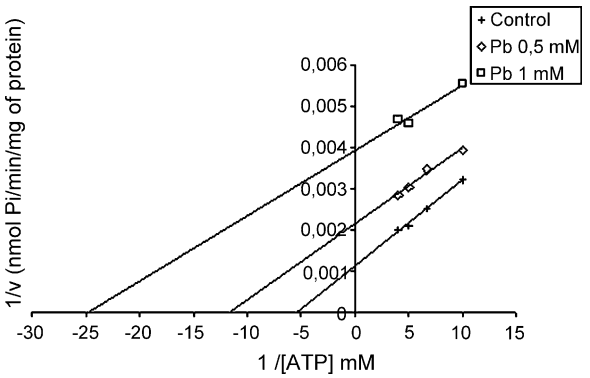

Fig. 5. Lineweaver–Burk plot of ATP hydrolysis and its interaction with Pb^{2+}. The concentration of the nucleotides varied from 0.1 to 0.250 mM in absence (+) and in the presence of 0.5 mM (◇) and 1 mM (□) of Pb^{2+}. The experiment was repeated six times and similar results were obtained. The data showed represents a typical experiment.

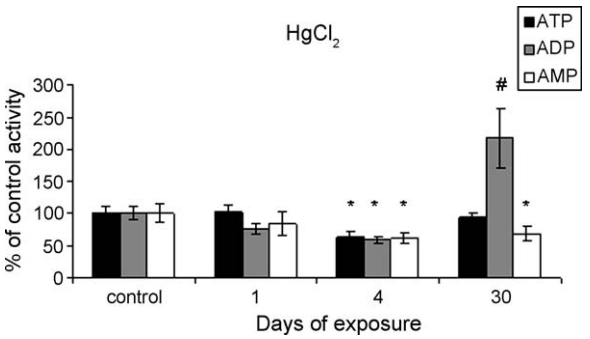

Fig. 6. In vivo effect of Hg^{2+} on nucleotide hydrolysis in zebrafish brain membranes. Bars represent mean ± S.D. of four independent experiments. Control specific activities for ATP, ADP and AMP hydrolysis were 619.2 ± 59.6, 146.4 ± 15.3 and 23.5 ± 3.5 nmol Pi min^{-1} mg^{-1} protein, respectively. *Significantly different from control (without metal added) ($P < 0.05$). #Statistical difference in relation to control and group submitted to 96 h of exposure.

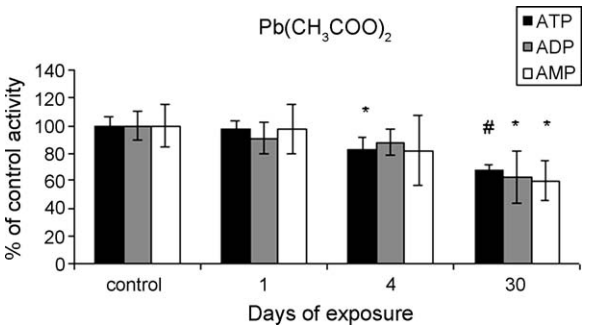

Fig. 7. In vivo effect of Pb^{2+} on nucleotide hydrolysis in CNS of zebrafish. Bars represent mean ± S.D. of four independent experiments. Control specific activities for ATP, ADP and AMP hydrolysis were 598 ± 35.7, 143.3 ± 14.8 and 22.4 ± 3.4 nmol Pi min^{-1} mg^{-1} protein, respectively. *Significantly different from control (without metal added) ($P < 0.05$). #Statistical difference in relation to control and group submitted to 96 h of exposure.

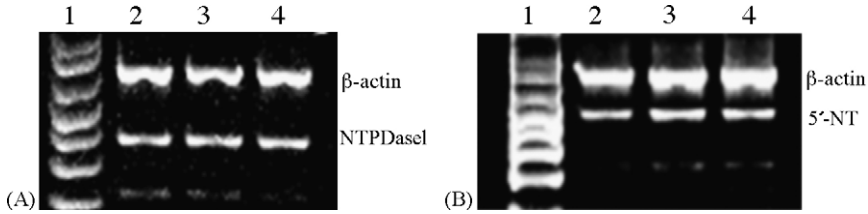

Fig. 8. Representative semi-quantitative RT-PCR mRNA for NTPDase1 (A) and 5′-nucleotidase (5′-NT) (B) from zebrafish brain after a chronic exposure (30 days) to 20 μg/L of Hg^{2+} and Pb^{2+}. (1) Represents the molecular weight marker (1 kb ladder Invitrogen); (2) control group; (3) Pb^{2+}-treated group; (4) Hg^{2+}-treated group. The PCR product of 380 bp for NTPDase1 and 433 bp for 5′-nucleotidase were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV. A β-actin control giving a fragment of 678 bp is presented.

Exposure to mercury or lead caused differential effect on nucleotide hydrolysis in brain membranes of zebrafish. After 24 h of exposure to mercury chloride, there were no significant changes on nucleotide hydrolysis (Fig. 6). The exposure to mercury chloride during 96 h caused a significant inhibition of ATP (37%), ADP (42%) and AMP (39%) hydrolysis (Fig. 6). Interestingly, after 30 days of exposure to mercury chloride, ATP hydrolysis return to the control level and ADP hydrolysis was strongly increased (118%) when compared to the control values. AMP hydrolysis at this time of exposure remaining inhibited (32%).

There were no significant changes on nucleotide hydrolysis after exposure to lead acetate during 24 h. After 96 h of exposure, it is possible to observe a significant decrease on ATP hydrolysis (17%), but not on ADP and AMP hydrolysis (Fig. 7). The chronic exposure to lead acetate during 30 days also promoted a significant inhibition of ATP (33%), ADP (37%) and AMP (40%) hydrolysis in brain membranes of zebrafish.

To verify if the chronic exposure to mercury or lead during 30 days was able to modify ecto-nucleotidases expression, semi-quantitative RT-PCR experiments were performed. There were no effects on NTPDase1 (Fig. 8A) and 5′-nucleotidase (Fig. 8B) at expression level of mRNA in zebrafish brain.

4. Discussion

This study reveals that in vitro and in vivo of exposure to $HgCl_2$ and $Pb(CH_3COO)_2$ promoted significant changes on nucleotide hydrolysis in zebrafish brain membranes. During acute and chronic treatments, no changes on swimming patterns and mortality were observed between control and treated groups at the concentration tested. Our results are consistent with Gonzalez et al. (2005) that reported no changes on behavior and mortality of zebrafish exposed to low doses dietary methylmercury (MeHg) concentrations up to 13.5 $\mu g\, g^{-1}$ of dry wet food for 63 days. Other papers showed no differences in the survival of other fish species exposed to chronic dietary MeHg (Drevnick and Sandheinrich, 2003; Houck and Cech, 2004).

For in vitro experiments, the results observed in the double-reciprocal plot indicated that $HgCl_2$ inhibited NTPDase activity in an uncompetitive manner and ecto-5′-nucleotidase activity in a non-competitive manner. For the in vivo treatments, we have tested an environmental low concentration of $HgCl_2$ and $Pb(CH_3COO)_2$, which corresponds to the dose found in aquatic environment (Berzas Nevado et al., 2003; Jha et al., 2003). After 96 h of exposure, $HgCl_2$ caused an inhibition on ecto-nucleotidase activities. However, after chronic treatment (30 days), ATP hydrolysis returned to the control level, ADP hydrolysis presented a dramatic increase and AMP hydrolysis remaining inhibited in brain membranes of zebrafish. This process can be due to an overcompensation response of the NTPDase to these pollutants. Thus, the exposure to low concentrations of these metals in the aquatic environment interferes with the extracellular nucleotide hydrolysis differing with the time and level of exposure. Moretto et al. (2004) have shown that treatment with subcutaneous injections of low doses of mercury chloride (0.1 mg/kg; 30 doses/30 days) promote activation of NTPDase and acetylcholinesterase activities, but not on 5′-nucleotidase in synaptosomes from cerebral cortex of rats.

Our results have shown that $Pb(CH_3COO)_2$, in a dose-dependent response, inhibited only the ATP breakdown in an uncompetitive manner. The in vivo exposure to this metal significantly decreased only ATP hydrolysis after 96 h. However, chronic exposure during 30 days promoted significant inhibition of ATP, ADP and AMP hydrolysis. In the literature, the evaluation of erythrocyte pyrimidine 5′-nucleotidase has been used as a toxicological parameter to occupational exposure to lead (Kim et al., 2002). Our study presents the first evidence about the inhibitory effect of this heavy metal on ecto-nucleotidase pathway after both short and long-term exposure.

The direct inhibitory effect of mercury and lead on NTPDase and 5′-nucleotidase was only observed at high concentrations when compared to doses tested at in vivo treatment. Our in vitro experiment with DTT suggests that the metals can bind to the enzyme, since DTT recover the nucleotidase activity. A possible direct effect of these metals on the enzyme activities after in vivo treatment cannot be discarded, since brain is able to accumulate large quantities of heavy metals (Tiffany-Castiglion and Qian, 2001; Gonzalez et al., 2005).

Furthermore, the effects observed after in vivo treatment have leaded us to investigate a possible indirect mechanism able to affect ecto-nucleotidases after exposure to these contaminants. Previous studies have shown that environmental pollutants, including heavy metals, are known to modulate protective measures, such as induction of xenobiotic-metabolizing enzymes, signal transduction pathways and oxidative stress response. These modifications can occur via transcriptional or post-translational modifications of important molecules in these pathways (Carvan et al., 2000; Nihei et al., 2001).

Therefore, we performed semi-quantitative RT-PCR experiments against NTPDase1 (a member that hydrolyzes ATP and ADP equally well) and ecto-5′-nucleotidase. However, our results indicate that the significant alterations observed on NTPDase1 and ecto-5′-nucleotidase activities after 30 days of treatment with the heavy metals probably were not related to changes in the expression levels.

Since there is no regulation of NTPDase1 and ecto-5′-nucleotidase at transcriptional level after chronic metals treatment, we analyzed the protein sequence in order to identify possible post-translational modifications that could be involved in the modulation of the enzymes. Wink et al. (2000) demonstrated that NTPDase can be detected as a phosphoprotein in different rat brain preparations, which could have implications in the regulation of this enzyme. It has been shown that the inactivation of ecto-ATPase activity promoted by ATP is reverted by alkaline phosphatase in rat brain synaptosomes, which suggests that these effects are mediated by phosphorylation (Martín-Romero et al., 1996). Furthermore, it has been reported that the activation of Protein Kinase C activates ecto-5′-nucleotidase in canine myocardium (Kitakaze et al., 1997). NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (http://www.cbs.dtu.dk/services/Netphosh), was used to analyze zebrafish NTPDase1 and ecto-5′-nucleotidase protein sequences (Blom et al., 2004). The results obtained from NTPDase1 sequence indicate the residues Ser151, Thr154 and Thr484 as three potential Protein Kinase C phosphorylation sites with high prediction scores (0.8, 0.87 and 0.8, respectively). Ecto-5′-nucleotidase reveals two putative Protein Kinase C phosphorylation sites (Ser59 and Thr206) and one cAMP-dependent protein Kinase phosphorylation site (Ser403) with high prediction scores (0.82, 0.84 and 0.81, respectively). Studies have shown that heavy metals, as mercury, lead and methyl mercury inhibited the PKC activity at micromolar concentrations (Rajanna et al., 1995). Further studies are required to evaluate a possible modulation exerted by heavy metals on PKC and their influence on kinetic behavior of nucleotide-metabolizing enzymes in central nervous system of zebrafish.

Alterations in several neurotransmission systems can explain some neurotoxicological characteristics of the heavy metals. These substances affect many transmission systems, like glutamatergic (Nihei and Guilarte, 2001), gabaergic (Lasley and Gilbert, 2002), serotoninergic (Oudar et al., 1989), dopaminergic (Faro et al., 2001) and cholinergic (Mirzoian and Luetje, 2002) systems. Many studies have demonstrated the coliberation and reciprocal modulation between ATP and other neurotransmitters systems (Burnstock, 2004). Extracellular nucleotides are important messenger both in physiological as well in pathological conditions. After its release in the synaptic cleft, ATP can be catabolized to ADP, AMP and adenosine. Adenosine has a strong neuroprotective effect, contrasting with the excitatory effect triggered by ATP (Kato et al., 2004). Studies have demonstrated that purines at high concentrations can induce cytotoxic effects (Chow et al., 1997). The effect that nucleotides have on cells depends on the extracellular catabolism mediated by ecto-nucleotidases, which regulate the concentration of ATP/adenosine and the response mediated by P2/P1 receptors, respectively. Therefore, based on the data presented herein, this study demonstrated that Hg^{2+} and Pb^{2+} affect the ecto-nucleotidase activities, an important enzyme pathway for the control of purinergic signaling. Further studies will be required to understand the role of purinergic system on the neurotoxicity promoted by heavy metals.

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