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Influence of mercury chloride on adenosine deaminase activity and gene expression in zebrafish (*Danio rerio*) brain

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ABSTRACT

Mercury is a widespread environmental contaminant that is neurotoxic even at very low concentrations. In this study we investigated the effects of mercury chloride on soluble and membrane adenosine deaminase (ADA) activity and gene expression in zebrafish brain. Inhibition of ADA activity was observed in the soluble fraction at 5–250 μM $HgCl_2$ (84.6–92.6%, respectively), whereas inhibition occurred at 50–250 μM in membrane fractions (20.9–26%, respectively). We performed *in vitro* experiments with chelants (EDTA and DTT) to test if these compounds prevented or reversed the inhibition caused by $HgCl_2$ and found that the inhibition was partially or fully abolished. The effect on ADA activity in soluble and membrane fractions was evaluated after acute (24 h) and subchronic (96 h) *in vivo* exposure of zebrafish to 20 μg/l $HgCl_2$. ADA activity in the soluble fraction was decreased after both acute (24.5%) and subchronic (40.8%) exposures, whereas in brain membranes the enzyme was inhibited only after subchronic exposure (21.9%). Semiquantitative RT-PCR analysis showed that $HgCl_2$ did not alter ADA gene expression. This study demonstrated that ADA activity was inhibited by mercury and this effect might be related to the neurotoxicity of this heavy metal.

1. Introduction

Heavy metals, such as mercury, are ubiquitous pollutants that are introduced in the aquatic environment by anthropogenic sources, mainly by industrial effluents (Rai, in press). Exposure to mercury has a wide range of deleterious effects on the aquatic and terrestrial biota even at sublethal concentrations. For animals and humans, the highly toxic effect of mercury, acting mainly on the central nervous system (CNS), its persistence in the environment and its accumulation in the food chain make it extremely hazardous (Järup, 2003).

Nucleotides and nucleosides have important roles in energetic and signaling systems in the CNS in both physiological and pathological conditions. Adenosine can be rapidly obtained by ATP breakdown via ectonucleotidase activities, such as nucleotide pyrophosphatase/phosphodiesterases (NPP), nucleoside triphosphate diphosphohydrolases (NTPDases), and 5′-nucleotidase, or it can be released from any cell when the intracellular concentration rises (Fredholm, 2002; Yegutkin, 2008). Extracellularly, adenosine acts as a neuromodulator, controlling both inhibitory and excitatory synapses by acting on G-protein coupled receptors (A_1, A_{2A}, A_{2B}, A_3) (Burnstock, 2007). Extracellular adenosine concentrations can be regulated by neural cell uptake through bi-directional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase, or deamination to inosine by adenosine deaminase (ADA) (Fredholm et al., 2005; Rosemberg et al., 2007a). ADA (E.C.3.5.4.4) is an enzyme which catalyzes the hydrolytic deamination of adenosine to inosine both in the cytosol and at the cell membrane (Franco et al., 1997; Rosemberg et al., 2008). Furthermore, studies have shown that extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007).

The zebrafish (*Danio rerio*) is a small teleost widely used in toxicological and biochemical studies (Rubinstein, 2006; Senger et al., 2006b). This fish combines the relevance of a vertebrate with the scalability of an invertebrate (Lieschke and Currie, 2007). Studies from our laboratory demonstrated the presence of NTPDases, 5′-nucleotidase, and adenosine deaminase activities

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in zebrafish brain (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008). In addition, we have shown that exposure to mercury chloride and lead acetate changes NTPDase and ecto-5′-nucleotidase activities in the central nervous system of this animal model (Senger et al., 2006a). Our group has also reported the differential expression pattern of ADA-related genes in zebrafish tissues, including brain, confirming that these genes (*ada1*, *ada2a*, *ada2b*, and *adal*) are present in this species (Rosemberg et al., 2007a). In goldfish, Western blot analysis of membrane preparations revealed the presence of a band of 35 kDa corresponding to the adenosine A_1 receptor, while both cytosol and membranes produced a band of 43 kDa corresponding to ADA (Beraudi et al., 2003).

Considering our previous work showing that exposure to mercury for 4 and 30 days inhibits NTPDases and ecto-5′-nucleotidase (Senger et al., 2006a), the aim of this study was to test the effect of mercury chloride on other enzymes involved in nucleoside metabolism, specifically ADA gene expression and activity in soluble and membrane fractions of zebrafish brain.

2. Materials and methods

2.1. Chemicals

Adenosine, EGTA, EDTA, Coomassie Blue G, and bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenol and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). 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 (Rio de Janeiro, Brazil). Cadmium acetate [$Cd(CH_3COO)_2$, CAS number 543-90-8], copper sulfate ($CuSO_4$, CAS number 7758-99-8), potassium dichromate ($K_2Cr_2O_7$, CAS number 7778-50-9), and cobalt chloride ($CoCl_2$, CAS number 7646-79-9) were purchased from Nuclear (São Paulo, Brazil). Zinc chloride ($ZnCl_2$, CAS number 7648-85-7) was purchased from QM (Rio de Janeiro, Brazil). Manganese chloride ($MnCl_2$, CAS number 7791-18-6) was purchased from Vetec Química Fina LTDA (Rio de Janeiro, Brazil). All reagents used were of high analytical grade.

2.2. Experimental animals

Adult zebrafish (*D. rerio*) were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50-l aquarium filled with continuously aerated, unchlorinated water. The temperature was kept at $25 \pm 1\,^{\circ}C$ under a 14/10-h light–dark photoperiod. Animal feeding and maintenance were done according to Westerfield (2000). The use and maintenance of zebrafish were according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (1996) and the Canadian Council on Animal Care guidelines on the care and use of fish in research, teaching and testing (2005).

2.3. In vitro assays

Mercury chloride (1–250 μM) was added to the reaction medium before preincubation with the enzyme and maintained throughout the enzyme assays. The experiments testing the effects of cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulfate, cobalt chloride, and manganese chloride were performed in a similar manner. Each metal was added to the reaction medium at 1, 5, 10, 50, 100, 250 μM; these concentrations were chosen according to previous studies showing the effect of these metals on other enzymes involved in nucleoside/nucleotide metabolism (Aikawa et al., 1980; Kundu et al., 1995; Senger et al., 2006a,b; Rosemberg et al., 2007b).

To verify if $HgCl_2$ could bind directly to the enzyme, we performed *in vitro* experiments with DTT and EDTA. Adenosine deamination was measured in the absence or presence of 5 and 250 μM $HgCl_2$ for soluble fractions or 250 μM $HgCl_2$ for membrane fractions in the reaction medium described in Section 2.6. The following groups were formed for both soluble and membrane fractions: (i) control, no $HgCl_2$ added; (ii) $HgCl_2$, addition of 5 μM (soluble fraction) or 250 μM $HgCl_2$ (soluble and membrane fractions); (iii) E + DTT, the fractions were preincubated for 5 min in the reaction mixture before DTT (500 μM) was added, then incubated for a further 5 min; (iv) DTT + E, the reaction medium was preincubated for 5 min with DTT (500 μM) before the fractions (E) were added, then incubated for a further 5 min; (v) E + EDTA, the fractions (E) were preincubated for 5 min in the reaction mixture before EDTA (500 μM) was added, then incubated for a further 5 min; (vi) EDTA + E, the reaction medium was preincubated for 5 min with EDTA (500 μM) and for a further 5 min with the fractions (E). Adenosine deaminase activity was determined as described below.

2.4. In vivo assays

The animals were introduced to the test aquariums (10 l) containing a solution of $HgCl_2$ at a final concentration of 20 μg/l, which has been reported in the aquatic environment (Berzas Nevado et al., 2003; Jha et al., 2003; Ram et al., 2003). The animals were maintained in the test aquarium for 24 and 96 h for acute and subchronic exposures, respectively.

2.5. Preparation of soluble and membrane fractions

Zebrafish were cryoanesthetized and immediately euthanized by decapitation, and whole brains were initially homogenized in 20 volumes (v/w) of chilled phosphate buffered saline (PBS) 2 mM EDTA, 2 mM EGTA, pH 7.4, in a glass-Teflon homogenizer in order to obtain both cellular fractions. Brain membranes were prepared according to a method described previously (Barnes et al., 1993) with minor modifications. The homogenate was centrifuged at $800 \times g$ for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at $40\,000 \times g$. The resulting supernatant and pellet corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS once and centrifuged for 20 min at $40\,000 \times g$. This freeze–thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used for biochemical assays. The material was maintained at 2–4 °C throughout preparation.

2.6. Determination of adenosine deaminase activity

Adenosine deaminase activity was determined using a Berthelot reaction as previously reported (Weisman et al., 1988). 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 the assays with 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 adenosine to a final concentration of 1.5 mM. The reaction was stopped by the addition of 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) after incubation for 75 min (soluble fraction) or 120 min (membrane fraction). Controls with the addition of the enzyme preparation after mixing with the phenol-nitroprusside reagent were used to correct for non-

enzymatic hydrolysis of substrates. The reaction mixtures were immediately added 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 ammonia produced was quantified by a colorimetric assay at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nmol of NH_3 min^{-1} mg $protein^{-1}$.

2.7. Protein determination

Protein was measured using Coomassie Blue as the color reagent (Bradford, 1976). A protein standard curve was prepared using bovine serum albumin (Stoscheck, 1990).

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

The expression of ADA-related genes *ada1*, *ada2a*, *ada2kb*, and *adal* was analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. To standardize the RNA extraction, all animals were euthanized at the same time of day (9:00–10:00 am). Total RNA from zebrafish brain was isolated using the Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. Afterwards, all samples were adjusted to 160 ng/μl and cDNA species were synthesized using SuperScript III™ First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. The *β-actin* primers were used as described previously (Chen et al., 2004). Primer sequences of ADA-related genes were designed and RT-PCR conditions were chosen according to Rosemberg et al. (2007a). The experimental conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of band intensities analyzed. PCR products were separated on a 1.0% agarose gel with GelRed 10× and visualized with ultraviolet light. The fragment lengths expected for the PCR reactions were confirmed using Low DNA Mass Ladder and *β-actin* was determined as an internal standard. Band intensities were analyzed by optical densitometry using the software ImageJ 1.37 for Windows after running all PCR products in a single gel.

2.9. Statistical analysis

All experiments were carried out in duplicate and means ± S.D. of at least three independent experiments are presented. Data were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test was employed where results achieved significance. *p*-Values ≤0.05 were considered as significant.

3. Results

The *in vitro* effect of $HgCl_2$ (1–250 μM) was observed on ADA activity in soluble and membrane fractions of zebrafish brain. In soluble fractions, inhibition was observed in the range 5–250 μM $HgCl_2$ (84.6–92.6%, respectively) (Fig. 1A). In membrane fractions, inhibition varied from 20.9–26% at 50 to 250 μM $HgCl_2$, respectively (Fig. 1B). We also tested the *in vitro* effect of cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulfate, cobalt chloride, and manganese chloride in soluble and membrane fractions and there were no significant changes in ADA activity in the presence of these metals (data not shown).

Our experiments demonstrated that the inhibition caused by 5 μM $HgCl_2$ was totally and partially blocked when DTT was added

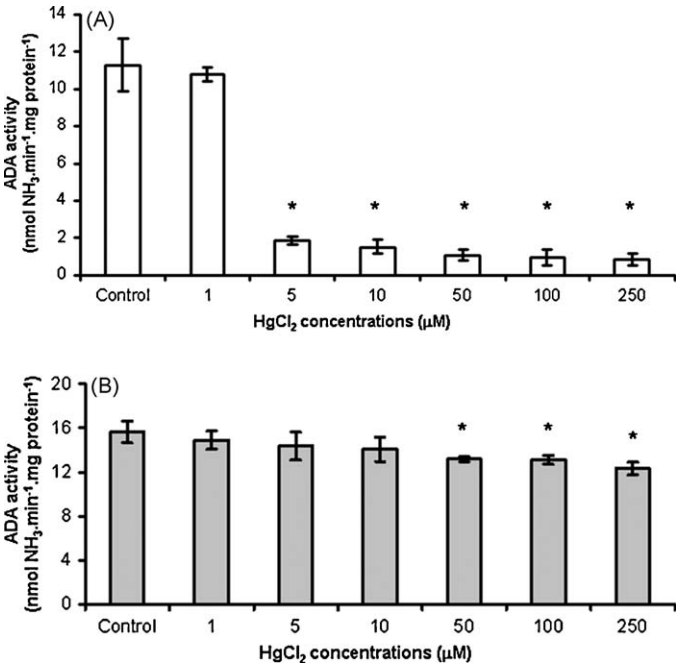

Fig. 1. Effect of mercury chloride concentrations on soluble (A) and membrane (B) preparations from zebrafish brain. Data represent means ± S.D. of three different replicate experiments. * represents a significant difference from control group (ANOVA, followed by Tukey test as post hoc, $p \leq 0.05$).

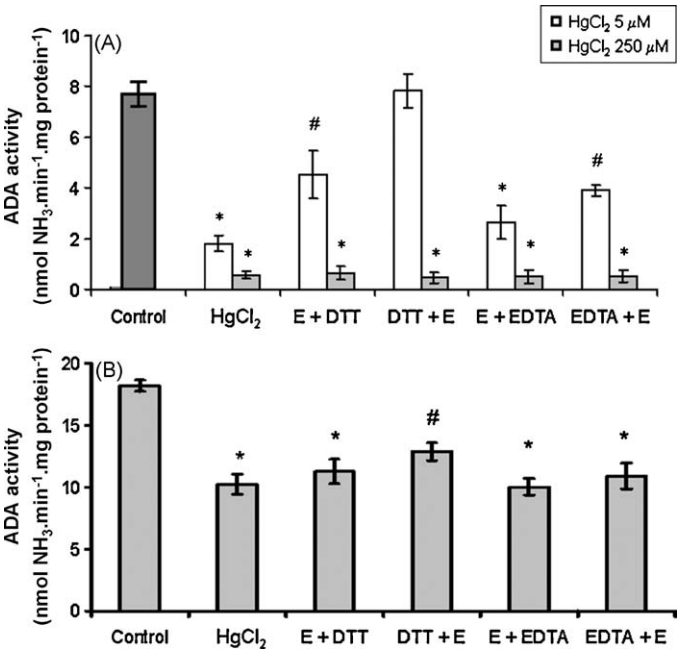

Fig. 2. Effect of DTT and EDTA on the inhibition caused by mercury chloride of zebrafish brain ADA activity in soluble fractions at 5 and 250 μM $HgCl_2$ (A) and membrane fractions at 250 μM $HgCl_2$ (B). Adenosine deamination was measured in the absence (control group) or in the presence of 5 or 250 μM $HgCl_2$ (as shown in the graph), preincubated for 5 min with DTT or EDTA (500 μM) and then incubated for a further 5 min with the enzyme preparations (E) (groups DTT + E and EDTA + E); or preincubated for 5 min with the enzyme preparations and then incubated for a further 5 min with DTT or EDTA (500 μM) (groups E + DTT and E + EDTA). Data represent means ± S.D. of four different replicate experiments. * represents a significant difference from control group (ANOVA, followed by Tukey test as post hoc, $p \leq 0.05$). # represents a significant difference from control and $HgCl_2$ group (ANOVA, followed by Tukey test as post hoc, $p \leq 0.05$).

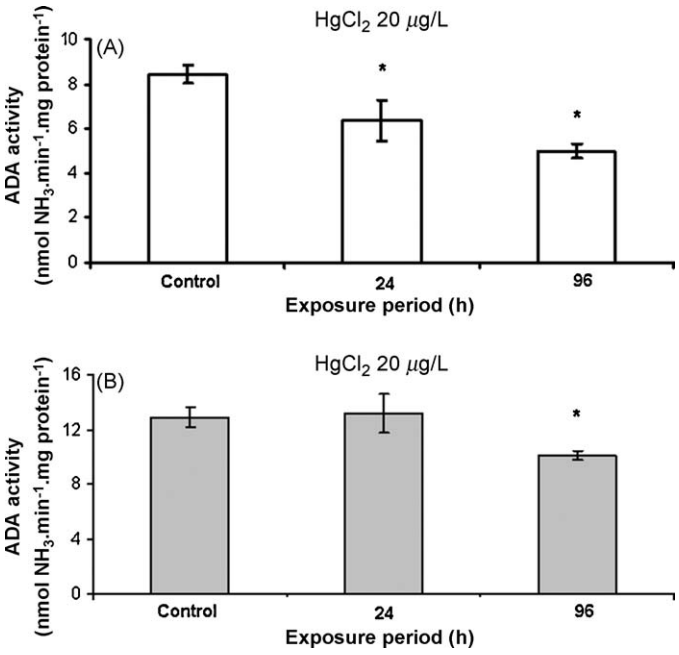

Fig. 3. Effect of acute (24 h) and subchronic (96 h) mercury chloride (20 μg/l) exposure on soluble (A) and membrane bound (B) ADA activity from zebrafish brain. Data represent means ± S.D. of at least four different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post hoc, $p \leq 0.05$).

before and after the soluble fraction, respectively. EDTA partially recovered the ADA activity in the soluble fraction only when added before the enzyme (Fig. 2A). Neither DTT nor EDTA prevented the inhibition caused by 250 μM $HgCl_2$ in the soluble fraction. For membrane fractions, only DTT partially blocked the $HgCl_2$ inhibition when added before the enzyme preparation (Fig. 2B).

The effect of $HgCl_2$ on ADA activity following acute (24 h) and subchronic (96 h) exposure of zebrafish to 20 μg/l $HgCl_2$ was evaluated in soluble (Fig. 3A) and membrane fractions (Fig. 3B). Significant mortality was not observed in control and treated groups, with the different exposure times to $HgCl_2$, and no changes in the swimming behavior were observed between control and treated groups (data not shown). The ADA activity in the soluble fraction was inhibited after both acute (24.5%) and subchronic

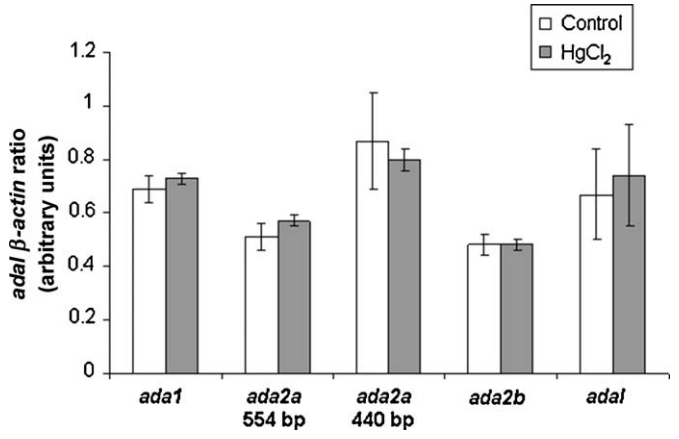

Fig. 4. ADA gene expression patterns after subchronic (96 h) mercury exposure (20 μg/l). The figure shows *β-actin*, *ada1*, *adal*, *ada2a*, and *ada2b* expression in brain of zebrafish. The results were expressed as optical densitometry (O.D.) of ADA-related genes versus *β-actin* expression. Data represent means ± S.D. of four different replicate RT-PCR experiments.

(40.8%) exposure times, whereas the membrane ADA activity was inhibited only after subchronic exposure to $HgCl_2$ (21.9%). Semiquantitative RT-PCR experiments were performed to verify whether $HgCl_2$ treatment could alter the expression of zebrafish brain ADA-related genes and the band densitometry using enzyme/β-actin ratios was determined for each treatment. The results did not show an effect of $HgCl_2$ on ADA gene expression in zebrafish brain (Fig. 4).

4. Discussion

The results presented herein demonstrate the influence of $HgCl_2$ on ADA activity and gene expression in zebrafish brain. $HgCl_2$ was able to inhibit ADA activity in soluble and membrane fractions. Our experiment with DTT and EDTA demonstrated that the inhibition was partially or fully abolished by these chelating agents, suggesting that $HgCl_2$ binds to the enzyme structure, altering adenosine deamination in zebrafish brain. The concentration used for *in vivo* $HgCl_2$ treatment was based on studies reporting that the concentration in the aquatic environment was around 20 μg/l (Berzas Nevado et al., 2003; Jha et al., 2003; Ram et al., 2003). Such studies document the concentration of mercury in waters, sediments and bivalves of the aquatic system historically impacted by mine wastes, large-scale industrialization, and the indiscriminate release of effluents.

There are few studies in the literature about the effect of metals on ADA activity. The influence of divalent cations on the structure and function of murine ADA has been previously demonstrated (Cooper et al., 1997). The authors showed that a single zinc or cobalt cofactor is bound to a high affinity site deep within the substrate-binding cleft and it is required for catalytic function. Furthermore, metal ions bound at additional sites inhibited the enzyme, and zinc, cobalt, copper, manganese, and cadmium were able to inhibit the ADA activity at micromolar concentrations. The decrease in ADA activity promoted by these added metals was immediately reversed by addition of EDTA to the reaction solution, indicating that they might be bound at readily accessible sites in the enzyme (Cooper et al., 1997). Our results show that a significant effect was obtained with 5 μM $HgCl_2$ for the soluble preparation. This is in the range of concentrations allowed by Brazilian legislation for salt water (Conselho Nacional do Meio Ambiente, resolution 357, 17/03/2005; www.mma.gov.br/port/conama/res/res05/res35705.pd). Although this dose did not have an acute toxic effect on aquatic organisms detected by ecotoxicological studies, it remains possible that long-term exposure to $HgCl_2$ at this concentration leads to a change in the adenosine pool, which can influence the neuroprotective effects produced by this nucleoside. In our study, the chelants reversed the inhibition of soluble and membrane bound ADA activity caused by mercury, which leads us to suggest that the addition of EDTA or DTT attenuates the inhibitory effect over a range of concentrations, because these compounds effectively reduce the concentration of free $HgCl_2$. In addition, analysis of the sequences of ADA-related genes in zebrafish tissues demonstrated the presence of –SH groups (Rosemberg et al., 2007a), which are believed to be the main target for mercury ions (Broniatowski and Dynarowicz-Łatka, 2009).

The ADA activity in the soluble fraction was significantly decreased after acute and subchronic exposures while the membrane ADA activity was inhibited only after subchronic exposure. The RT-PCR results showed that $HgCl_2$ did not interfere in the expression of ADA-related genes, indicating that this metal did not modulate the mRNA synthesis of enzymes able to deaminate adenosine. Therefore, it is possible to suggest that the significant decrease in adenosine deamination promoted by $HgCl_2$ *in vivo* might be due to a direct effect on enzyme activities, as

verified in the *in vitro* experiments. It is important to mention that the inhibition of ADA activity in soluble and membrane fractions observed with $HgCl_2$ *in vitro* did not present a dose-response effect, such that increasing doses did not produce significantly more severe effects. A similar effect of $HgCl_2$ was observed on ATP and ADP hydrolysis due to NTPDase activities, in which the inhibition observed at 0.05–0.25 mM $HgCl_2$ did not present a concentration-dependent response (Senger et al., 2006a).

In previous studies in our laboratory we performed a phylogenetic analysis and found the presence of ADA1, ADA2, and ADAL in zebrafish brain (Rosemberg et al., 2007a). It has been suggested that the presence of these enzymes could be contributing in a different manner to the regulation of adenosine/inosine levels in distinct cellular fractions of porcine and zebrafish brains (Kukulski et al., 2004; Rosemberg et al., 2007a). Traditionally, ADA1 has been considered a cytosolic enzyme, constituting 90–100% of the total intracellular ADA activity (Franco et al., 2007; Iwaki-Egawa and Watanabe, 2002). There is evidence that cell-surface ADA1 is co-localized with adenosine A_1, A_{2B} receptors and concentrative nucleoside transporters, suggesting that this enzyme might be also involved in a fine tuning modulation of purinergic signaling (Saura et al., 1998; Herrera et al., 2001; Hirsh et al., 2007). No differences have so far been found between intracellular ADA1 and ecto-ADA1 regarding catalytic activity or molecular characteristics (Franco et al., 2007). The coexistence between ADA1 and the membrane bound enzyme 5′-nucleotidase has been shown in subcellular fractions of cultured brain cells and rat brain synaptosomes (Trams and Lauter, 1975; Franco et al., 1986). In addition, a previous study demonstrated that, together with ADA1, ADA2 also contributes to adenosine deamination in the extracellular milieu, thus belonging to a new family of growth factors with ADA activity (Zavialov and Engström, 2005). Rosemberg et al. (2007a) have shown that zebrafish possess ADA2 sequences, suggesting that this enzyme may also play a role in the cleavage of adenosine in the extracellular space in this species. Although no evidence related to ADAL functionality and location has been presented so far, the presence of conserved amino acids involved in adenosine deamination, its similarity with ADA1 and the presence of *adal* mRNA transcripts in zebrafish brain (Rosemberg et al., 2007a) might indicate a putative role for this enzyme in cleaving adenosine. Considering that the inhibition of adenosine deamination in the soluble fraction caused by $HgCl_2$ was more pronounced that observed in the membrane ADA activity, it is possible to suggest a differential subcellular location of these ADA-related enzymes in zebrafish brain and a distinct effect of $HgCl_2$ on these enzyme family members. Moreover, these aspects might be also important to explain the absence of effect on total adenosine deamination in soluble and membrane fractions after treatments with cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulfate, cobalt chloride, and manganese chloride.

Extracellular nucleotides and nucleosides are important signaling molecules that require effective mechanisms for their signal regulation (Yegutkin, 2008). This regulation is exerted by a broad range of nucleotide-degrading and interconverting extracellular enzymes (Zimmermann, 2006; Abbracchio et al., 2009). Adenosine deamination is an important mechanism able to promote tissue homeostasis and adenosine signaling in brain (Franco et al., 1997; Sun et al., 2005). Furthermore, the product of this reaction, inosine, has signaling properties in brain, where it acts on A_3 adenosine receptors (Shen et al., 2005). We have already demonstrated that NTPDase and 5′-nucleotidase activities are inhibited by toxic elements such as mercury, lead, zinc, cadmium, and copper (Rosemberg et al., 2007b; Senger et al., 2006a,b). Although the inhibitory effect of $HgCl_2$ on ADA activity indicates a possible decrease in inosine synthesis, it might be an important mechanism to increase the levels of the neuromodulator adenosine since previous data have shown that different lengths of exposure to this metal decrease nucleotide hydrolysis in zebrafish brain membranes (Senger et al., 2006a).

Taken together, this study demonstrated that mercury inhibited the activity of ADA, an important enzyme in purinergic metabolism, suggesting that adenosine/inosine levels could be altered by this metal. These findings suggest that both extracellular and intracellular purine metabolism is modulated by $HgCl_2$ and this could be involved in the neurotoxic effects of mercury.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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