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Acute and subchronic copper treatments alter extracellular nucleotide hydrolysis in zebrafish brain membranes

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Abstract

Copper is a divalent cation with physiological importance since deficiency of copper homeostasis can cause serious neurological diseases. ATP is an important signalling molecule stored at nerve endings and its inactivation is promoted by ecto-nucleotidases. In this study, we verified the effect of acute and subchronic copper treatments on ecto-nucleotidase activities in zebrafish brain membranes. Treatment with copper sulfate (15 μg/L) during 24 h inhibited ATP hydrolysis (16%), whereas ADP and AMP hydrolysis were not altered. Nevertheless, a 96-h exposure with the copper concentration mentioned above inhibited NTPDase (31% and 42% for ATP and ADP hydrolysis, respectively) and ecto-5′-nucleotidase (40%) activities. NTPDase1, NTPDase2_mg and NTPDase2_mv transcripts were decreased after copper exposures during 24 and 96 h. Subchronic copper treatment also reduced the NTPDase2_mq and ecto-5′-nucleotidase expression. *In vitro* assays demonstrated that NTPDase activities were reduced after copper exposure during 40 min. ATP hydrolysis was inhibited at 0.25, 0.5 and 1 mM (13%, 31% and 48%, respectively) and ADP hydrolysis also had a significant decrease at these same copper concentrations (41%, 63% and 68%, respectively). In contrast to the subchronic exposure, no significant changes on ecto-5′-nucleotidase were observed after *in vitro* assays. Lineweaver–Burk plots suggested that both inhibitory effects on nucleotide hydrolysis may occur in a non-competitive manner. Altogether, these findings indicate that copper is able to promote distinct changes on ecto-nucleotidases after *in vivo* and *in vitro* treatments and, consequently, it could control the nucleotide and nucleoside levels, modulating the purinergic signalling.

Keywords: Copper; Ecto-nucleotidases; NTPDase; Ecto-5′-nucleotidase; Nucleotide hydrolysis; Zebrafish

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1. Introduction

Extracellular ATP is a ubiquitous signalling molecule, which acts in the purinergic system as neurotransmitter and/or neuromodulator at nerve endings (Burnstock, 2000; Cunha and Ribeiro, 2000). It can be co-released at synaptic cleft with several neurotransmitters, including acetylcholine, noradrenaline and serotonin (Rathbone et al., 1999) and its biological effects are mediated by ionotropic P2X and metabotropic P2Y receptors (Ralevic and Burnstock, 1998). The inactivation of ATP signalling is promoted by cell-surface enzymes named ecto-nucleotidases, which include NTPDases and ecto-5′-nucleotidase. The NTPDase (nucleoside triphosphate diphosphohydrolase) family is able to promote ATP/ADP hydrolysis at different rates and the ecto-5′-nucleotidase cleaves AMP to adenosine (Rathbone et al., 1999; Zimmermann, 2001). This nucleoside acts as an important neuromodulator through specific G protein-coupled receptors named A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001).

The purinergic system has been investigated in zebrafish, which has several characteristics making it a useful vertebrate model to study many biological processes including development and chemical toxicity (Linney et al., 2004; Senger et al., 2005, 2006; Rico et al., 2006). The genome of this fish shares many similarities with the human genome (Barbazuc et al., 2000). Ionotropic P2X receptors have already been identified and characterized in this specie (Kucenas et al., 2003) and GABAergic neurons were described in adult zebrafish brain (Kim and Macdonald, 2003). Studies have recently demonstrated the presence of NTPDases (Rico et al., 2003) and ecto-5′-nucleotidase (Senger et al., 2005) in brain membranes of zebrafish. Although there is no evidence about zebrafish NTPDase3 and NTPDase8 orthologous so far, it was demonstrated that this teleost presents three distinct NTPDase2 forms (Rico et al., 2006). Furthermore, it is interesting to study the effect of metal exposure in zebrafish, because it can uptake metals directly from water and elevated concentrations can be accumulated in CNS (Grosell and Wood, 2002).

Copper is an essential element involved in many metabolic pathways. It is an important co-factor for several enzymes, such as metalloenzymes, including cytochrome *c* oxidase and Cu/Zn superoxide dismutase (Kako et al., 2004; Wang et al., 2005). Copper is obtained from diet sources, chelated by amino acids, absorbed in small intestine and transported in the blood in a binding form (Gaetke and Chow, 2003). Evidence demonstrated that copper can be found in CNS tightly bound for catalytic use by cuproproteins (Schlief and Gitlin, 2006). Copper may also be loosely bound to small molecules such as histidine and glutathione, representing an available pool for releasing and acting as a functional neuromodulator in CNS (Mathie et al., 2006; Schlief and Gitlin, 2006). High concentrations of copper in the brain can be found in synaptic vesicles, particularly in hippocampus, olfactory bulb and locus coeruleus, being released after electrical stimuli in the micromolar range (Ono and Cherian, 1999; Mathie et al., 2006).

Studies have shown that copper has a dualistic function on synaptic activity. It can form a complex binding to AMPA/kainate receptors in cultured rat cortical neurons and, possibly, the co-release of copper with glutamate is important to modulate excitatory synapses (Weiser and Wienrich, 1996). In addition, copper also promotes the inhibition of $GABA_A$ receptor currents in a α subunit-dependent manner controlling the fast inhibitory synaptic neurotransmission (Kim and Macdonald, 2003). Deficiency of copper levels in central neurons induces severe neurological disorders as Menkes disease (Kreuder et al., 1993). Wilson disease, other pathology related to copper metabolism, is a rare autosomal recessive inherited disorder characterized by excessive deposition of copper in several tissues (Brewer and Yusbasiyan-Gurkan, 1992). Copper ions may induce the production of reactive oxygen species (ROS) and the oxidative stress is one of the major effects of cellular copper accumulation (Bongarzone et al., 1995).

It has been demonstrated that modifications of copper homeostasis in CNS could be involved in neuronal cell death (Levenson, 2005). Copper also modulates purinoceptors P2X by a non-competitive inhibition of ATP-evoked currents in a metal-binding site (Virginio et al., 1997; Coddou et al., 2003). Moreover, there is evidence that apoptosis events can be mediated by extracellular ATP signalling through ionotropic $P2X_7$ receptors (Lepine et al., 2006).

Considering that (i) zebrafish is a model widely used in toxicological and neurochemical studies; (ii) copper can play a key role on brain homeostasis modulating the P2 purinoceptor signalling; (iii) there is no data relating to copper exposure and nucleotide catabolism in CNS, the goal of this study was to investigate whether this metal can influence the inactivation of ATP signalling, mediated by the ecto-nucleotidase pathway. In this sense, we evaluated the acute (24 h), subchronic (96 h) and *in vitro* effects of copper on ecto-nucleotidase activities in zebrafish brain. In addition, the influence of copper on NTPDases and ecto-5′-nucleotidase genes expression was also investigated.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*), weighting 0.250–0.450 g, were obtained from commercial suppliers and kept for at least a week before the experiments in a 50-L thermostated aquarium filled with unchlorinated water constantly aerated. Fish were kept at 26 ± 2 °C under a natural light–dark photoperiod and fed twice a day with a commercial fish pellet. The use of animals was according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

Trizma Base, Malachite Green, polyvinyl alcohol, ammonium molybdate, nucleotides, EGTA, EDTA, sodium citrate, Coomassie Blue G, bovine serum albumin, magnesium, and calcium chloride were purchased from Sigma (St. Louis, MO, USA). Copper, added as pentahydrate copper sulfate ($CuSO_4 \cdot 5H_2O$, CAS number: 7758-99-8), was purchased from Nuclear (Brazil) and all other reagents used were of analytical grade.

2.3. In vivo and in vitro experiments

For *in vivo* treatment, the animals were introduced in a 20-L test aquarium 15 min after the addition of 15 μg/L copper sulfate, a concentration reported in previous studies with rainbow trout (Dethloff et al., 1999). This copper concentration was lower than estimated LC_{10} for zebrafish (Oliveira-Filho et al., 2004) and was chosen to ensure fish survival and to avoid changes in the swimming pattern in metal-treated fish. After acute (24 h) or subchronic (96 h) exposure, fish were sacrificed, the brains excised and membranes were prepared. Copper sulfate was tested at 0.05–1 mM for *in vitro* assays, in order to evaluate high concentrations of this metal which could be attained during neurodegenerative diseases (White and Cappai, 2003). Selected concentrations were added to reaction medium before preincubation with the enzyme and maintained throughout the enzyme assays.

2.4. Membrane preparation

Brain membranes were prepared according to the method described previously by Barnes et al. (1993). Whole zebrafish brains were initially homogenized in 60 volumes (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4 adjusted 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 centrifuged for 20 min at 40,000 × *g*. This freeze–thaw-wash procedure was used to ensure the lysis of the membranes. The final pellet was resuspended and used for biochemical assays. The material was maintained at 2–4 °C throughout preparation.

2.5. Ecto-nucleotidase assays

NTPDase (Rico et al., 2003) and ecto-5′-nucleotidase (Senger et al., 2004) assays were performed as described previously. Brain membranes of zebrafish (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 ecto-5′-nucleotidase activity) in a final volume of 200 μL. All samples were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 1 mL of a colorimetric reagent composed by 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% Malachite Green was added (Chan et al., 1986). After 20 min, the quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm. 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 was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

2.6. Protein determination

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

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

Zebrafish NTPDase1, three different forms of NTPDase2 (NTPDase2_mg, NTPDase2_mq, NTPDase2_mv) (Rico et al., 2006) and β-actin (Chen et al., 2004) primers were used as described previously. Ecto-5′-nucleotidase sequence was obtained from GenBank and used to design specific primers. The optimal annealing temperatures were also tested (Table 1).

TRIzol® reagent (Invitrogen) was employed to isolate total zebrafish brain RNA in accordance with manufacturer instructions. RNA was quantified spectrophotometrically and 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 were performed as described previously (Rico et al., 2006). The amplification for different NTPDase2, 5′-nucleotidase 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). PCR conditions for NTPDase1 were similar as described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR

Table 1
PCR primer design and annealing conditions

Enzymes	Sequences (5′–3′)	PCR product (bp)	Annealing temperature (°C)	GenBank Accession number
NTPDase1	CCCATGGCACAGGCCGGTTG (forward), GCAGTCTCATGCCAGCCGTG (reverse)	380	54	AAH78240
NTPDase2_mg[a]	GGAAGTGTTTGACTCGCCTTGCACG (forward), CAGGACACAAGCCCTTCCGGATC (reverse)	554	64	XP_697600
NTPDase2_mq[a]	CCAGCGGATTTAGAGCACGCTG (forward), GAAGAACGGCGGCACGCCAC (reverse)	313	64	XP_687722
NTPDase2_mv[a]	GCTCATTTAGAGGACGCTGCTCGTG (forward), GCAACGTTTTCGGCAGGCAGC (reverse)	263	64	AAH78419
5′-Nucleotidase	ACCTCCGAGGAGTGTCGCTTTCG (forward), CCTTGTTGGGGACCAGCGGTTC (reverse)	433	54	NP_957226
β-Actin	GTCCCTGTACGCCTCTGGTCG (forward), GCCGGACTCATCGTACTCCTG (reverse)	678	54	AAC13314

[a] Correspond to the two first amino acids residues of the protein sequence.

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. A negative control was included for each set of PCR reactions. PCR products were analyzed on 1.5% agarose gel, containing ethydium bromide and visualized with ultraviolet light. The β-actin gene was amplified for normalization and the Invitrogen 1 kb Plus DNA ladder was used as molecular marker in order to confirm the fragment size. 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.

2.8. Statistical analyses

Data obtained from *in vivo* assays were expressed as mean ± S.D. and analyzed by Student's *t*-test. The *in vitro* experiments data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan multiple test range as post hoc. The value of $P \leq 0.05$ was considered as significant.

3. Results

The acute and subchronic effects of copper exposure were evaluated on ATP, ADP and AMP hydrolysis in zebrafish brain membranes. After acute exposure to 15 μg/L copper, ATPase activity was significantly inhibited (16%), but no changes were observed on ADPase and AMPase activities (Fig. 1A). Nevertheless, the subchronic treatment caused a significant decrease on ATP (31%), ADP (42%) and AMP (40%) hydrolysis when compared to untreated fish (Fig. 1B).

Semi-quantitative RT-PCR experiments were conducted to verify whether copper treatment could alter

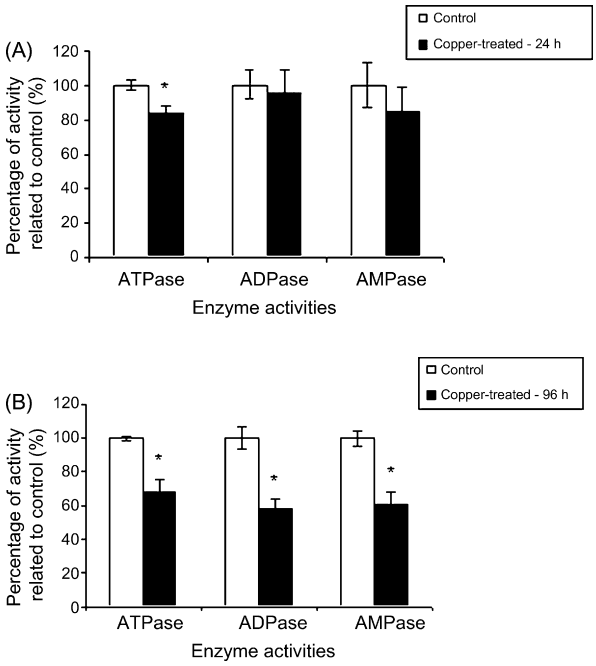

Fig. 1. Nucleotide hydrolysis in zebrafish brain membranes after acute (24 h) (A) and subchronic (96 h) (B) 15 μg/L copper exposures. The graphs show the percentage of specific activity related to control group ± S.D. of, at least, three different experiments. For acute treatment, the control ATPase, ADPase and AMPase activities were 514 ± 15, 124 ± 10 and 22 ± 3 nmol Pi min^{-1} mg^{-1} of protein, respectively. For subchronic exposure, the ATPase, ADPase and AMPase activities were 546 ± 6, 124 ± 8 and 21 ± 1 nmol Pi min^{-1} mg^{-1} of protein, respectively. *Significantly different from control group ($P \leq 0.05$, Student's *t*-test).

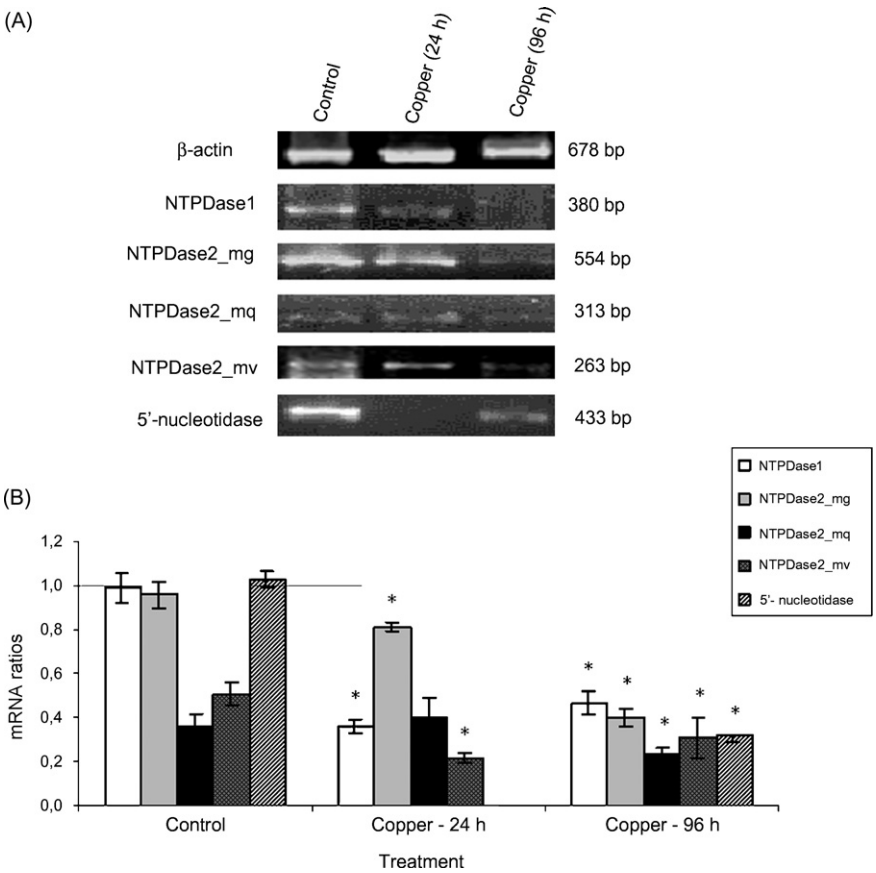

Fig. 2. Effect of acute (24 h) and subchronic (96 h) copper exposure on ecto-nucleotidases transcripts. The figure shows β-actin, NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv and 5′-nucleotidase mRNA expression in adult zebrafish (A) and the enzyme/β-actin mRNA ratios obtained by optical densitometry analysis (B). Three independent experiments were performed, with entirely consistent results. *Significantly different from control group ($P \leq 0.05$, ANOVA followed by Duncan post hoc).

the expression of zebrafish brain ecto-nucleotidases (Fig. 2A) and the enzyme/β-actin mRNA ratios were determined for each treatment (Fig. 2B). Since acute copper exposure did not promote any significant changes on AMP hydrolysis, the ecto-5′-nucleotidase gene expression was not evaluated after this treatment. The results have shown that NTPDase1, NTPDase2_mg and NTPDase2_mv transcript levels decreased after acute and subchronic copper exposures when compared to control group. A significant decrease on NTPDase2_mq and ecto-5′-nucleotidase expression was observed after subchronic treatment.

In order to evaluate a direct inhibitory effect on ecto-nucleotidase activities, we have tested *in vitro* copper concentrations varying from 0.05 to 1 mM. NTPDase activities decreased significantly when compared to control. Copper, at 0.25, 0.5 and 1 mM, produced an inhibition of ATP hydrolysis (13%, 31% and 48%, respectively) (Fig. 3A). A similar inhibitory effect was also detected on ADP hydrolysis (Fig. 3B) at the copper concentrations mentioned above (41%, 63% and 68%, respectively), whereas copper did not change ecto-5′-nucleotidase activity (Fig. 3C). Lineaweaver–Burk double reciprocal plots were analyzed within the range (0.1–0.25 mM) of ATP and ADP concentrations in the absence and presence of copper (Fig. 4). The results suggested that ATP (Fig. 4A) and ADP (Fig. 4B) inhibition may occur in a non-competitive manner. Thus, copper would be able to significantly modify the V_{max} but not K_M. The V_{max} values for ATPase activity in the absence or presence of 0.5 and 1.0 mM copper were: 750.9 ± 31.7 ($r = 0.9858$), 589.6 ± 34.7 ($r = 0.9976$), and 453.2 ± 63.3 ($r = 0.9911$) nmol Pi min^{-1} mg^{-1} of protein, respectively. For ADPase activity, the V_{max} values in the absence or presence of 0.5 and 1.0 mM copper were: 265.5 ± 4.1 nmol Pi min^{-1} mg^{-1} ($r = 0.9531$), 208.5 ± 7.7 nmol Pi min^{-1} mg^{-1} ($r = 0.9757$), and 142.3 ± 5.1 nmol Pi min^{-1} mg^{-1} ($r = 0.9639$) of protein, respectively. For each nucleotide, the V_{max} values in the absence or presence of copper concentrations

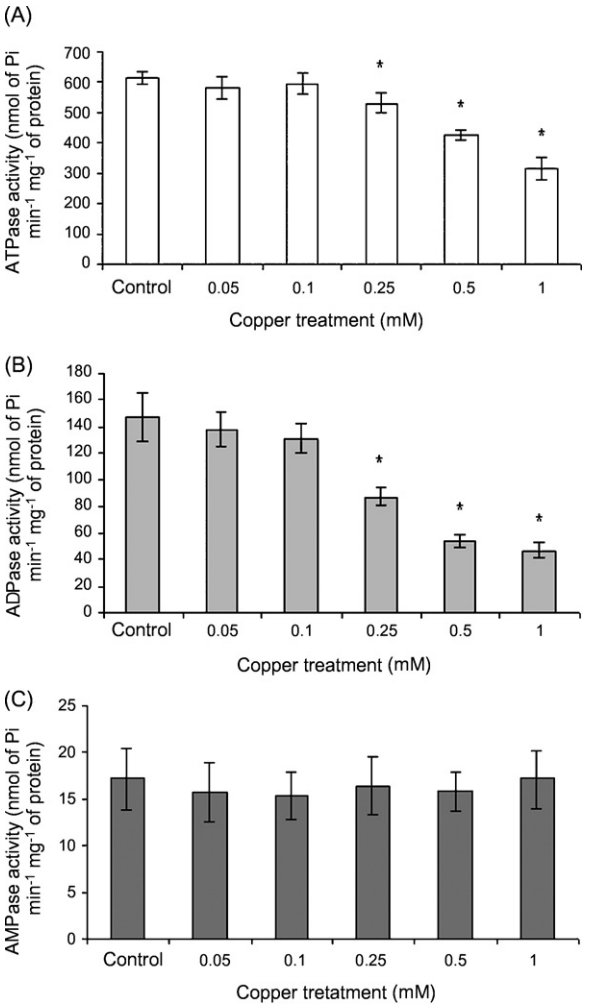

Fig. 3. *In vitro* effect of different concentrations of copper (0.25–1 mM) on ATP (A), ADP (B) and AMP (C) hydrolysis in zebrafish brain membranes. Bars represent the mean ± S.D. of three different experiments. The control activities (without copper) were 614 ± 21, 147 ± 18 and 17 ± 3 nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP and AMP hydrolysis, respectively. *Significantly different from control group ($P \leq 0.05$, ANOVA followed by Duncan post hoc).

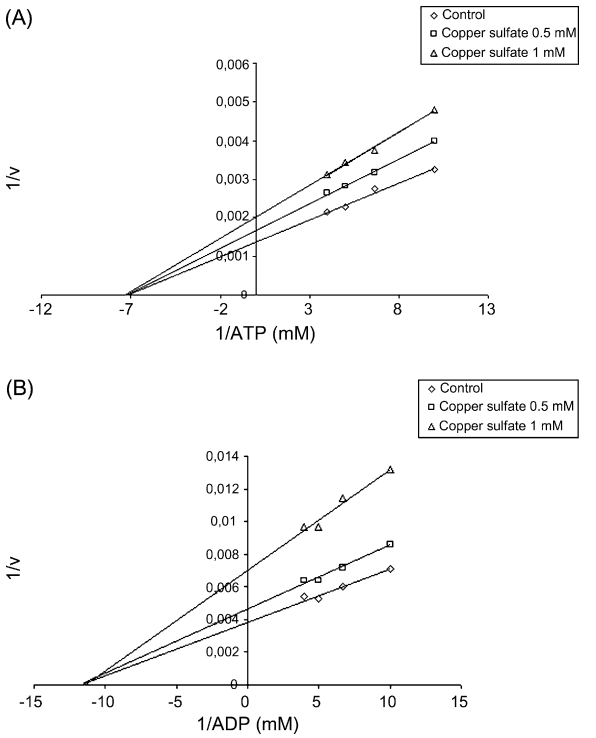

Fig. 4. Kinetic analysis of NTPDase inhibition by copper in zebrafish brain membranes. The graphs show double-reciprocal plots of NTPDase activity for ATP (A) and ADP (B) concentrations (0.1–0.25 mM) in the absence (◊) and in the presence of 0.5 mM (□) and 1 mM of copper (△). All experiments were repeated three times and similar results were obtained.

tested were significantly different by ANOVA followed by Duncan test as post hoc ($P < 0.01$).

4. Discussion

The present study has shown that copper is able to alter ecto-nucleotidase activities in zebrafish brain membranes. Fish exposed to 15 μg/L copper during 24 h (acute treatment) showed a significant decrease on ATP hydrolysis, whereas no effects were observed on ADP and AMP hydrolysis. Subchronic treatment (96 h) significantly inhibited nucleotide hydrolysis, decreasing both NTPDase and ecto-5′-nucleotidase activities.

The changes promoted by copper on nucleotide hydrolysis in zebrafish brain may be possible due to distinct mechanisms. Here we have evaluated two possible actions of copper in CNS: (i) alterations on NTPDase and ecto-5′-nucleotidase expression; (ii) inhibitory effect on ecto-nucleotidases by direct mechanisms.

The expression of NTPDases in zebrafish brain was evaluated after acute and subchronic copper treatments. Due to the absence of significant effect on AMP hydrolysis after acute treatment, the ecto-5′-nucleotidase gene expression was not evaluated in this condition. The 24-h exposure promoted a decrease on NTPDase1, NTPDase2_mg, and NTPDase2_mv mRNA levels. Moreover, the expression patterns of NTPDase2_mq and ecto-5′-nucleotidase transcripts were also reduced after subchronic treatment. Previous study has shown the effect of copper on gene expression, demonstrating that this metal may be able to regulate the transcriptional activity of human p53 protein (Tassabehji et al., 2005). Therefore, the lower NTPDase1, NTPDase2_mg and NTPDase2_mv after both treatments and the inhibition of NTPDase2_mq and ecto-5′-nucleotidase expression

after a 96-h copper exposure could be one of the mechanisms involved in the inhibition of nucleotide hydrolysis in zebrafish brain membranes.

Other processes may also explain the inhibitory effect observed on ecto-nucleotidase activities. It has been demonstrated that copper is a metal that can induce lipid peroxidation (Pinchuk et al., 1998), which is able to alter membrane fluidity. This phenomenon could partially contribute to explain the toxicity associated with this metal in ecto-enzymes (Garcia et al., 2005). In addition, copper may also induce proteolysis, which was verified in previous studies involving the chaperone CopZ of *E. hirae* (Lu and Solioz, 2001; Solioz, 2002). Since NTPDases1–3, 8 and ecto-5′-nucleotidase are enzymes tightly bound to plasma membrane, it is not possible to discard a copper-stimulated oxidative damage or even proteolysis on zebrafish brain ecto-nucleotidases.

Studies have shown that copper is able to act as an allosteric modulator of ionotropic $P2X_4$ (Coddou et al., 2005). Furthermore, copper can act on $P2X_7$ purinoceptors, most likely by modulation of the agonist binding site, resulting in a decreased agonist affinity for this receptor (Virginio et al., 1997). The ecto-nucleotidase pathway is responsible to maintain the concentrations of extracellular purine and pyrimidine nucleotides in physiological conditions. Evidence has demonstrated that upregulation of NTPDase (CD39) activity decreased the extent of apoptosis triggered by P2X receptors in response to high concentrations of extracellular ATP (Goepfert et al., 2000; Lepine et al., 2006). Thus, our data suggest that alterations on nucleotide catabolism could be a target for copper-induced cytotoxicity. A decrease on ecto-nucleotidase activities associated with the modulation of P2X receptors might be a possible mechanism to explain the neurodegenerative effects promoted by copper.

Copper can be found in different parts of CNS and it is accumulated in brain as other physiological metals, such as zinc, with relative importance for synaptic mechanisms (Kim and Macdonald, 2003). It has been known that copper concentrations in cerebrospinal fluid have been estimated to be around 70 μM and the normal extracellular copper concentrations in brain ranged from 0.2 to 1.7 μM (Mathie et al., 2006). During neurodegenerative diseases, copper can be released at synaptic cleft reaching extremely high concentrations (200–400 μM) (White and Cappai, 2003). In order to establish a possible direct effect of copper on ecto-nucleotidase activities, we performed *in vitro* assays at concentrations varying from 0.05 to 1 mM copper. This metal inhibited NTPDase activities at 0.25 to 1 mM and Lineweaver–Burk double-reciprocal plots suggested that inhibition of ATP and ADP hydrolysis may occur in a non-competitive manner after copper exposure for 40 min. On the other hand, AMP hydrolysis did not change significantly. Since which copper species affecting the enzymes in each type of exposure (*in vivo* and *in vitro*) is unknown, the concentrations of copper and the exposing durations are quite different, it is not possible to ensure if the metal alters directly or indirectly the enzyme activities. It has been observed an inhibitory effect on AMP hydrolysis after subchronic copper exposure, whereas ecto-5′-nucleotidase did not present any significant change after *in vitro* experiments. Therefore, it is possible to suggest that copper may be regulating this enzyme activity *in vivo* at transcriptional level as it has been evidenced by the decrease of ecto-5′-nucleotidase mRNA transcripts detected in RT-PCR assays.

This study investigated the effects of acute and subchronic copper treatments on NTPDase and ecto-5′-nucleotidase activities and expression in zebrafish brain. The inhibition on ecto-nucleotidase activities and a decrease on NTPDase and ecto-5′-nucleotidase transcripts could be involved in the neurotoxic effects induced by copper in CNS.

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References

Barbazuc, W.B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J.A., McPherson, J.D., Johnson, S.L., 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res. 10, 1351–1358.

Barnes, J.M., Murphy, P.A., Kirkham, D., Henley, J.M., 1993. Interaction of guanine nucleotides with [^{3}H] kainate and 6-[^{3}H] cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J. Neurochem. 61, 1685–1691.

Bongarzone, E.R., Pasquini, J.M., Soto, E.F., 1995. Oxidative damage to proteins and lipids of CNS myelin produced by *in vitro* generated reactive oxygen species. J. Neurosci. Res. 41, 213–221.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 218–254.

Brewer, G.J., Yusbasiyan-Gurkan, V., 1992. Wilson disease. Medicine 71, 139–164.

Burnstock, G., 2000. Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin. Med. 2 (1), 45–53.

Chan, K., Delfert, D., Junguer, K.D., 1986. A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal. Biochem. 157, 375–380.

Chen, W.-Y., John, J.A.C., Lin, C.-H., Lin, H.-F., Wu, S.-C., Lin, C.-H., Chang, C.-Y., 2004. Expression of metallothionen gene during embryonic and early larval development in zebrafish. Aquat. Toxicol. 69, 215–227.

Coddou, C., Morales, B., Huidobro-Toro, J.P., 2003. Neuromodulator role of zinc and copper during prolonged ATP applications to $P2X_4$ purinoceptors. Eur. J. Pharmacol. 472 (1–2), 49–56.

Coddou, C., Lorca, R.A., Acuna-Castilo, C., Grauso, M., Rassendren, F., Huidobro-Toro, J.P., 2005. Heavy metals modulate the activity of the purinergic $P2X_4$ receptor. Toxicol. Appl. Pharmacol. 2002 (2), 121–131.

Cunha, R.A., Ribeiro, J.A., 2000. ATP as a presynaptic modulator. Life Sci. 68, 119–137.

Dethloff, G.M., Schlenk, D., Khan, S., Bailey, H.C., 1999. The effects of copper on blood and biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). Arch. Environ. Contam. Toxicol. 36, 415–423.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N., Linden, J., 2001. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 53, 527–552.

Gaetke, L.M., Chow, C.K., 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189, 147–163.

Garcia, J.J., Martinez-Ballarin, E., Millan-Plano, S., Allue, J.L., Albendea, C., Fuentes, L., Escanero, J.F., 2005. Effects of trace elements on membrane fluidity. J. Trace Elem. Med. Biol. 19 (1), 19–22.

Goepfert, C., Imai, M., Brouard, S., Csizmadia, E., Kaczmarek, E., Robson, S.C., 2000. CD39 modulates endothelial cell activation and apoptosis. Mol. Med. 6 (7), 591–603.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Kako, K., Takehara, A., Arai, H., Onodera, T., Takahashi, Y., Hanagata, H., Ogra, Y., Takagi, H., Kodama, H., Suzuki, K.T., Munekata, E., Fukamizu, A., 2004. A selective requirement for copper-dependent activation of cytochrome *c* oxidase by Cox17p. Biochem. Biophys. Res. Commun. 324, 1379–1385.

Kim, H., Macdonald, R.L., 2003. An N-terminal histidine is the primary determinant of alpha subunit-dependent Cu^{2+} sensitivity of alphabeta3gamma2L GABA(A) receptors. Mol. Pharmacol. 64, 1145–1152.

Kreuder, J., Otten, A., Fuder, H., Tümer, Z., Tonnesen, T., Horn, N., Dralle, D., 1993. Clinical and biochemical consequences of copper-histidine therapy in Menkes disease. Eur. J. Pediatr. 152, 828–832.

Kucenas, S., Li, Z., Cox, J.A., Egan, T.M., Voigt, M.M., 2003. Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience 121, 935–945.

Lepine, S., Le Stunff, H., Lakatos, B., Sulpice, J.C., Giraud, F., 2006. ATP-induced apoptosis of thymocytes is mediated by activation of P2X7 receptor and involves *de novo* ceramide synthesis and mitochondria. Biochim. Biophys. Acta 1761 (1), 73–82.

Levenson, C.W., 2005. Trace metal regulation of neuronal apoptosis: from genes to behavior. Physiol. Behav. 86, 399–406.

Linney, E., Upchurch, L., Donerly, S., 2004. Zebrafish as a neurotoxicological model. Neurotoxicol. Teratol. 26, 709–718.

Lu, Z.H., Solioz, M., 2001. Copper-induced proteolysis of the CopZ copper chaperone of *Enterococcus hirae*. J. Biol. Chem. 276 (51), 47822–47827.

Mathie, A., Sutton, G.L., Clarke, C.E., Veale, E.L., 2006. Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol. Ther. 111 (3), 567–583.

Oliveira-Filho, E.C., Lopes, R.M., Paumgartten, F.J.R., 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56, 369–374.

Ono, S., Cherian, M.G., 1999. Regional distribution of metallothionein, zinc, and copper in the brain of different strains of rats. Biol. Trace Elem. Res. 69 (2), 151–159.

Pinchuk, I., Schnitzer, E., Lichtenberg, D., 1998. Kinetic analysis of copper-induced peroxidation of LDL. Biochim. Biophys. Acta 1389, 155–172.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Rathbone, M.P., Middlemiss, P.L., Gysbers, J.W., Andrew, C., Herman, M.A., Reed, J.K., Cicarelli, R., Di Iorio, P., Caciagli, F., 1999. Trophic effects of purines in neurons and glial cells. Prog. Neurobiol. 59, 663–690.

Rico, E.P., Senger, M.R., Fauth, M.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci. 73, 2071–2082.

Rico, E.P., Rosemberg, D.B., Senger, M.R., Arizi, M.D., Bernardi, G.F., Dias, R.D., Bogo, M.R., Bonan, C.D., 2006. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol. Teratol. 28 (4), 489–496.

Schlief, M.L., Gitlin, J.D., 2006. Copper homeostasis in the CNS: a novel link between the NMDA receptor and copper homeostasis in the hippocampus. Mol. Neurobiol. 33 (2), 81–90.

Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). Comp. Biochem. Physiol. 139B, 203–207.

Senger, M.R., Rico, E.P., Arizi, M.D., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2005. Carbofuran and Malathion inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes. Toxicology 212, 107–115.

Senger, M.R., Rico, E.P., Arizi, M.D., Frazzom, A.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2006. Exposure to Hg^{2+} and Pb^{2+} changes NTPDase and ecto-5′-nucleotidase activities in central nervous system of zebrafish (*Danio rerio*). Toxicology 226 (2–3), 229–237.

Solioz, M., 2002. Role of proteolysis in copper homoeostasis. Biochem. Soc. Trans. 30 (4), 688–691.

Tassabehji, N.M., VanLandingham, J.W., Levenson, C.W., 2005. Copper alters the conformation and transcriptional activity of the tumor suppressor protein p53 in human Hep G2 cells. Exp. Biol. Med. 230 (10), 699–708.

Virginio, C., Church, D., North, R.A., Surprenant, A., 1997. Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. Neuropharmacology 36 (9), 1285–1294.

Wang, J., Ma, J.H., Giffard, R.G., 2005. Overexpression of copper/zinc superoxide dismutase decreases ischemia-like astrocyte injury. Free Radic. Biol. Med. 38, 1112–1118.

Weiser, T., Wienrich, M., 1996. The effects of copper ions on glutamate receptors in cultured rat cortical neurons. Brain Res. 742, 211–218.

White, A.R., Cappai, R., 2003. Neurotoxicity from glutathione depletion is dependent on extracellular trace copper. J. Neurosci. Res. 71, 889–897.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Develop. Res. 52, 44–56.