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Neurobehavioral alterations plus transcriptional changes of the heat shock protein 90 and hypoxia inducible factor-1α in the crucian carp exposed to copper

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ABSTRACT

The various physiological alterations caused by copper (Cu) exposure in fish have attracted great interests toward neuronal strategies against Cu toxicity. Here, neurobehavioral activities (including anxiety-like behaviors) and transcriptional levels of heat shock protein (Hsp)90 and hypoxia inducible factor-1alpha (HIF-1α) were evaluated in the crucian carp (*Carassius carassius*) treated with nominal sub-lethal higher (1.45 mg/L) and lower (0.30 mg/L) concentrations of $CuCl_2$. Both concentrations accounted for diminished swimming speed and food intake plus a strong preference for the dark side of the light/dark apparatus together with a reduction of crossings between the two compartments with respect to controls. Contextually, Hsp90 and HIF-1α transcripts were largely down- and up-regulated, respectively, in some brain areas such as in the medial part of the dorsal telencephalon (Dm, −52%) and in the ventral part of the ventral telencephalon (Vv, +68%). When carps were transferred to $CuCl_2$-free water, some behaviors were rescued especially for fish previously exposed to 0.30 mg/L concentration. In this same condition, Hsp90 mRNA levels were recuperated (−94%) in the medial preglomerular nucleus (NPGm) with respect to exposed fish while HIF-1α mRNA was mostly down-regulated in telencephalic stations. Moreover, recovery capacities of this extraordinary resistant fish was exhibited by evident reductions (−80%) of the dark argyrophilic granules such as in the ventral telencephalon (VTel). Overall, our results point to interesting responses against Cu toxicity involving Hsp90 and HIF-1α transcripts that may constitute early indicators of environmental stressors leading to the repair of metal-induced damages in fish with notable brain plasticity properties.

1. Introduction

Copper (Cu) is an ubiquitous aquatic pollutant that reaches surface waters following intense agricultural, mining and industrial activities. A continuous exposure of fish to this heavy metal causes toxic effects (Kolmakov et al., 2009; Luzio et al., 2013) thereby modifying vital functions like swimming (Vieira et al., 2009) and feeding maneuvers (Kuz'mina, 2011). Cu targets the gills, where it causes mucus production, cell swelling and epithelial lifting thus interfering with gas exchange by increasing diffusion distance across the gill epithelium and consequently evoking tissue hypoxia (van Heerden et al., 2004; De Boeck et al., 2010). Following the different hazardous events, great attention has been recently focused on novel neuroreceptor strategies during toxicity processes (Zizza et al., 2014), as well as on protective and adaptive measures that allow fish to tolerate adverse environmental conditions (Giusi et al., 2012). Among the different factors involved with such measures, the heat shock protein (Hsp) 90 is recognized as a key molecular element of stressful states in fish deriving from changes of oxygen regimes (Stensløkken et al., 2010) plus aquatic pollutants (Eder et al., 2009; Zizza et al., 2014). It is widely known that molecular chaperones ensure protein-folding properties during physiological conditions thereby becoming determinant for cell health and organism longevity in response to stressors (Saibil, 2013). This is not so surprising since Hsp90 turns out to be a

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; ACS, amino cupric silver stain; Cu, copper; DIG, digoxigenin-11-dUTP; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; HIF-1α, hypoxia inducible factor-1alpha; Hsp90, heat shock protein 90; NDLI, diffuse nucleus of the inferior lobe; NPGm, medial preglomerular nucleus; OD, optical density; OT, optic tectum; TLo, torus longitudinalis; VCe, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; VTel, ventral telencephalon; Vv, ventral part of the ventral telencephalon.

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critical component of the cellular machinery that delivers α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors into the postsynaptic membrane (Gerges et al., 2004). Moreover, the biological role of Hsp90 is not only important in mammals but also in fish as pointed out by its transcriptional variations in the crucian carp following changes in water quality (An et al., 2014).

Target proteins of Hsp90 include transcription factors and many signal transduction proteins of different cellular pathways. In particular Hsp90, a molecular switchboard capable of controlling the fate of its clients, is at the crossroads of the late folding and early degradation trajectories (Karagöz and Rüdiger, 2015). Of the various factors, hypoxia inducible factor-1alpha (HIF-1α) mRNA levels resulted to be very high among the tissues (including the brain) that are important for hypoxic survival (Rytkönen et al., 2014). Consequently, HIF-1α is considered an useful biomarker of environmental hypoxia in fish (Kodama et al., 2012). It is already known that HIF-1α stabilization or degradation is strictly dependent on the competition between receptor of activated protein C kinase (RACK1) and Hsp90, which are essential elements of an O_2/prolyl hydroxylase/von-Hippel-Lindau-independent mechanism for regulating HIF-1α stability (Liu and Semenza, 2007). At the brain level, HIF-1α elicits protective measures against ischemic conditions and neurodegenerative disorders by up-regulating cell survival genes (Correia and Moreira, 2010; Singh et al., 2012). In addition, such an important indicator of stress accounts for heavy metal-dependent hypoxic-like conditions (Pan et al., 2013), although the precise role of HIF-1α on neuronal responses of fish exposed to heavy metals has yet to be defined.

On the basis of the above information, it was the aim of the present study to examine behavioral activities (feeding, swimming, resting plus anxiety-like behavior) and transcriptional levels of Hsp90 plus HIF-1α in the crucian carp (*Carassius carassius*) exposed to Cu and then transferred to metal-free water during a recovery test. The choice of this fish species was based on its extraordinary resistance to adverse environments, especially those characterized by low oxygen levels (Nilsson and Lutz, 2004). Such an adaptive feature makes the crucian carp an useful model for the evaluation of neurobehavioral strategies against stressful factors like metals. For this purpose, crucian carps were exposed to nominal sub-lethal higher (1.45 mg/L) and lower (0.30 mg/L) concentrations of $CuCl_2$ that fell within the range used for other freshwater teleosts (Atli et al., 2006; Kuz'mina, 2011). These concentrations, although being considerably lower than those of natural waters and fish food, were reported to eventually rise to drastic levels (177 mg/L) near industrial sites (Kuz'mina, 2011). Results of the present study may supply indications concerning Hsp90 and HIF-1α transcriptional activities during both Cu neurotoxicity and neurobehavioral recovery in such a hypoxia resistant fish. Moreover, the effects of environmentally relevant Cu concentrations during light/dark preference test may supply interesting insights regarding anxiety-like states of the crucian carp as main targets of Cu contamination.

2. Materials and methods

2.1. Maintenance of specimens

For this study, freshwater fish *C. carassius* (body weight = 5–8 g) were provided by a local farmer of Santa Caterina Albanese (CS) and then transported to our laboratory where they were left to acclimate for at least one week. The parameters for fish maintenance consisted of a 12 h light:12 h dark photoperiod in flow-through tanks containing constantly aerated and filtered dechlorinated tap water (150 L) at a temperature of 21–22 °C, pH 7 as well as dissolved oxygen 6.5–7.0 mg/L. During the entire experiment the presence of NO_2 and NO_3 were detected using commercial kit test strips (Tetra GmbH, Germany). Fish were daily fed at 10:15 am with commercial food (Friskies multigrain, Milano, Italy) corresponding to a 2% of wet body weight ration. Animal maintenance and experimental procedures were carried out in compliance with the ethical provisions for Care and Use of Laboratory Animals reported in the legislative law n°116 (27-01-1992) and authorized by the National Committee of the Italian Ministry of Health.

2.2. Metal exposure

After acclimation, fish were exposed for 2 days (d) to nominal sublethal higher (1.45 mg/L; $n = 16$) or lower (0.30 mg/L; $n = 16$) $CuCl_2$ (Sigma, Milan, Italy) concentrations and compared to controls ($n = 8$) that were never exposed to $CuCl_2$. The chosen concentrations corresponding to about 1/2 and 1/10, respectively, of the medial lethal concentration (2d-LC_{50}; 2.717 mg/L) fell more or less within the range used for other freshwater teleosts (Atli et al., 2006; Kuz'mina, 2011). LC_{50} value was previously established in a preliminary study (Table 1) by Probit analysis, using a specific software (Probit analysis, version 1.5) developed by EPA. This analysis permitted us to also verify that both higher and lower $CuCl_2$ concentrations did not fall within the range of lethal concentrations.

All toxicological treatments were conducted during a *Static renewal toxicity test* according to standard procedure guidelines (American Society for Testing Material, 1997) completely renewing this heavy metal concentration in water every 1d. It was not possible to measure Cu concentrations during our experiments but due to a Cu reduction of only 4–6% over a 4d exposure period, regardless of pH and temperature (Carvalho and Fernandes, 2006), we expected that within the short time period of 1d Cu concentrations would remain constant. Throughout metal exposure, aquaria were only equipped with an aerator without any chemical filters to avoid a reduction of the metal concentration and water parameters were constantly checked to ensure that they remained within the above ranges.

2.3. Recovery test

In order to verify if fish were able to recover from Cu-dependent alterations at the end of the exposure period (2d), some specimens from both 1.45 mg/L ($n = 8$) and 0.30 mg/L ($n = 8$) of $CuCl_2$ concentration groups plus controls were then transferred to new tanks containing metal-free water and left for other 2d. For this part, comparisons were carried out between fish recovered at 1d and 2d with respect to their controls and to those belonging to 2d of treatment, which corresponds with the end of metal exposure. Even during this test, water was changed regularly at 1d intervals to ensure the same experimental conditions of the

Table 1
Probit analysis for the determination of Cu LC_{50} in *Carassius carassius* .

Points		Exposure	95% Confidence limits	
		Concentration (mg/L)	Lower	Upper
LC	1.00	2.095	1.748	2.278
LC	5.00	2.261	1.976	2.414
LC	10.00	2.355	2.107	2.494
LC	15.00	2.420	2.199	2.549
LC	50.00	2.717	2.585	2.854
LC	85.00	3.050	2.896	3.352
LC	90.00	3.134	2.962	3.497
LC	95.00	3.264	3.059	3.728
LC	99.00	3.522	3.241	4.214