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ScienceDirect
Toxicology and Applied Pharmacology 227 (2008) 248–256
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Specific cerebral heat shock proteins and histamine receptor cross-talking mechanisms promote distinct lead-dependent neurotoxic responses in teleosts

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Received 25 July 2007; revised 19 September 2007; accepted 22 October 2007
Available online 1 November 2007

Abstract

Recent interests are beginning to be directed towards toxic neurobiological dysfunctions caused by lead (Pb) in aquatic vertebrates. In the present work, treatment with a maximum acceptable toxic concentration of this heavy metal was responsible for highly significant ($p<0.01$) abnormal motor behaviors such as hyperactive movements in the teleost *Thalassoma pavo* and the same treatment accounted for significantly ($p<0.05$) enhanced hyperventilating states. On the other hand, greater abnormal motor behaviors were detected in the presence of the histamine (HA) receptor subtype 2 (H_2R) antagonist cimetidine (Cim), as shown by the very robust ($p<0.001$) increases of the two behavioral states. Interestingly, elevated expression levels of stress-related factors, i.e. heat shock protein70/90 (HSP90/70) orthologs were reported for the first time in hypothalamic and mesencephalic areas of Pb-treated teleosts. In particular, an up-regulation of HSP70 was readily detected when this heavy metal was given concomitantly with Cim, while the histamine subtype 3 antagonist (H_3R) thioperamide (Thio), instead, blocked Pb-dependent up-regulatory trends of both chaperones in mostly hypothalamic areas. Moreover, intense neuronal damages of the above brain regions coincided with altered expressions of HSP70 and HSP90 when treated only with Cim. Overall these first results show that distinct H_nR are able to exert a net neuroprotective role arising from their interaction with chaperones in fish exposed to Pb-dependent stressful conditions making this a potentially key interaction especially for *T. pavo*, aquatic species which plays an important ecological role towards the survival of other commercially vital fishes.
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Keywords: Ornate wrasse; Motor behaviors; Chaperones; Heavy metal; Neuronal damage

Introduction

Environment pollutants such as heavy metals pose serious risks to many aquatic organisms by changing neurophysiologic, biochemical and behavioral parameters (Scott and Sloman, 2004). Of all heavy metals, lead (Pb), due to its high abundance and physical characteristics such as ductility plus a high density, accounts for some of the major threats to genomic processes in different vertebrates (Mortada et al., 2004). In aquatic habitats, Pb can be highly toxic for fish since it can disrupt metabolic pathways and cause ionoregulatory damages (Rogers et al., 2005). At the physiological level, Pb accumulates in cellular organelles (Qian and Tiffany-Castiglioni, 2003) impairing the properties of some calcium-dependent proteins such as heat shock proteins (HSPs) with lethal consequences on reproductive behaviors (Feder and Hofmann, 1999). It is well known that HSPs assist immature proteins to fold into the native conformation and can rescue them from their previous aggregate form (Beere, 2005). Studies have considered HSP90 as specialized chaperone targeting client proteins in signal transduction and neurotransmitter release (Gerges et al., 2004) that, together with other chaperones such as HSP70, exerts a protection against ischemic brain injuries (Giffard et al., 2004).

The ability of some HSPs to suppress multiple types of cell death has been highlighted in necrotic death and classical

Abbreviations: Cim, cimetidine; CP, central posterior thalamic nucleus; Dm, dorsomedial telencephalic region; DP, dorsal posterior thalamic nucleus; HA, histamine; HSP, heat shock protein; MAPKs, mitogen-activated protein kinases; MAT, maximum acceptable toxicant; NAT, anterior tuberal nucleus; NGa, anterior part of glomerolosus nucleus; NH, habenular nucleus; NLTm, medial part of lateral tuberal nucleus; NLTv, ventral part of lateral tuberal nucleus; NPGm, medial preglomerular nucleus; NPP, posterior periventricular nucleus; NRP, posterior recess nucleus; NSC, suprachiasmatic nucleus; Pb, lead; SGC, stratum griseum centralis; Thio, thioperamide; Tlo, torus longitudinalis; VCe, cerebellum valvula; VM, ventromedial thalamic nucleus; Vot, ventral optic tract.

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0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2007.10.018

apoptosis (Ravagnan et al., 2001), making these chaperones principal factors of brain neuroprotective processes. In view of Pb-induced neuronal damages, attention has been shifted to its influence on increased synthesis of certain biogenic amines that influence behavioral activities and overall circadian rhythms (Devi et al., 2005). The histamine (HA) neuroreceptor system is one of the major aminergic systems exerting key neurological functions such as arousal, movement and feeding via three pharmacologically distinct HA receptor subtypes denoted as $H_{1-3}R$ (Parson and Ganellin, 2006). Recently, *in vivo* exposure to heavy metals produced a significant increase of mitogen-activated protein kinases (MAPKs) with the consequent facilitation of HAergic-related brain development and neuroplasticity alterations (Kukko-Lukjanov et al., 2006; Leal et al., 2006).

It is thus our intention to investigate the toxic effects of Pb on some motor and feeding behaviors of a marine teleost, the ornate wrasse *Thalassoma pavo*. For the present study, the ornate wrasse constitutes a valuable model to study neuronal adaptive processes (Giusi et al., 2005a) as well as of the abnormal neurobehaviors induced by such a heavy metal. In order to establish the type of interaction of HSP70/90 in the abnormal behavioral responses under stressful conditions, the nucleotidic sequences of the two chaperone orthologs that are lacking in public databases for the ornate wrasse were identified and characterized for the first time in this teleost species. The expression of these chaperones was handled in the different diencephalic and extra-diencephalic (mesencephalon and telencephalon) areas that are noted for their control in feeding and motor behaviors in the fish (Kaslin et al., 2004). From the moment that heavy metals have shown to respond to HAergic activities, the influences of Pb toxic effects were also assessed on these behavioral activities in the presence and absence of specific antagonists for the two major HAergic subtypes and namely H_2R (cimetidine, Cim) or H_3R (thioperamide, Thio). In this context, the interaction of the above chaperones with these two HAergic neuroreceptor subtypes ($H_{2,3}R$) in the presence of heavy metals may provide interesting insights regarding the type of molecular mechanisms responsible for the promotion of stressful states in aquatic vertebrates.

Materials and methods

Animals and treatments. Local *T. pavo* (body weight 25–30 g; length 16–18 cm) were collected from Tyrrhenian Sea, acclimated in our laboratory in flow-through tanks (150 L of seawater, 19–21 °C, pH 7.8) and maintained under a 12 h light:12 h dark photoperiod. With the intention of knowing the physiological HSP70/90 neuronal brain levels plus the determination of whether their handling induced stressful conditions, other captured fishes were immediately decapitated and used as controls of outdoor environmental conditions (wild fishes) for only *in situ* hybridization analysis. Afterwards, some fishes ($n=4$) were exposed for 1 week to a sublethal (0.4 mg/l) or a maximum acceptable toxicant (MAT) dose (1.6 mg/l) of Pb ($PbNO_3$) dissolved in seawater. These doses were chosen on the basis of its 96-h LC_{50} (lethal median concentration) value (6 mg/L) plus on the range of ecologically relevant concentrations mimicking physiological modifications (Giusi et al., 2005b). Data obtained for the above conditions were compared to fishes maintained under the same conditions with the exception that seawater alone was added to the tanks of these controls ($n=4$) that were matched with all experimental groups.

In other tests, the influence of HA antagonists (Sigma, Milan, Italy) and namely H_2R (Cim) and H_3R (Thio) was assessed on Pb-treated *T. pavo*. For this part, fishes ($n=4$) were treated intraperitoneally (i.p.) for 1 week with biological active doses of Cim (200 mg/kg body weight) or Thio (10 mg/kg body weight)± a MAT concentration of Pb. These treatment groups were compared to animals that were exposed to a MAT Pb concentration, for this part considered as controls ($n=4$), which were maintained under the same experimental conditions to that of the treatment groups with the exception that they received via i.p. a physiological solution (i.p. 0.9% NaCl). Animal maintenance and experimental procedure were in accordance with the *Guiding Principles in the Use of Animals in Toxicology* and efforts were made to minimize animal suffering and reduce number of specimens used.

Behavioral analysis. The effects of sublethal and MAT Pb concentrations on motor and feeding behaviors of *T. pavo* were evaluated in four 1-h sessions each day for 1 week. In the same manner, the specific influence of the two HA antagonists on the above Pb concentrations was compared to Pb-treated animals. Throughout the behavioral sessions, feeding habits were checked to ensure that the fish ingested water containing the contaminant, even though it is known that the uptake of these compounds relies primarily on their passage through the gill system. For the behavioral study we analyzed feeding habits and motor activities (hyperactive states and bumping movements as well as hyperventilation). During the different feeding sessions, we recorded the frequency of attempts made by the ornate wrasse to ingest food, which was proportional to the quantity (milligrams) of food up taken in each session. These behaviors were recorded with a digital video camera (TR 7000 E, Sony; Tokyo, Japan) and the values, expressed as mean activity within a 24 h interval±s.e.m., were analyzed using a blind scoring system by four different observers. The elaboration of these data was handled using a specific software for behavioral analyses (EthoLog software-version 2.2.5, Visual Basic; Brazil). For this part the validity of the behavioral observations derived from an inter-rater reliability test of the different behavioral categories that supplied an intraclass correlation coefficient (ICC) of 0.75, which is very similar to the values calculated for other behavioral studies (Persons and Bertagnolli, 1999).

Histological analysis via Fluoro-Jade B staining method. Fluoro-Jade B staining methods were used to study degenerative processes since this fluorescein displays a selective affinity for damaged neurons (Schmued and Hopkins, 2000). Fishes were treated in the same manner as in the behavioral study, then they were decapitated and their brains were quickly removed and stored at −20 °C according to previous cryostat (Micron-H505 E, Zeiss, Germany) procedures (Canonaco et al., 1997). Serial brain sections (30μm) were next stored at 4 °C and after rinsing them in ethanol solutions (100% for 3 min; 70% for 3 min), these sections were next left in a permanganate solution 0.06% for 15 min. Afterwards, the brain sections were transferred to a highly specific Fluoro-Jade B staining solution (0.001%) that due to its highly photo sensitive property was left for 30 min in a dark room. These samples were used for a qualitative and quantitative analysis of damaged neuronal brain regions in all treatment groups (fluorescent microscopy, Leica; FITC filter) as previously reported (Anderson et al., 2005). For the estimation of the number of damaged neuronal fields in diencephalic and extra-diencephalic areas, it was necessary to calculate the volume (defined as Vref) of areas such as the preoptic area, hypothalamus, dorsal and ventral telencephalon, plus optic tectum using the following formula:

$$\mathrm{Nv} : \left[\sum (N/\mathrm{Vsection})/n\right] \times \mathrm{Vref}$$

In this case Nv represents the number of stained damaged neurons; N is the number of damaged neurons in a single section; Vsection is the volume of a single section; n is the number of sections; Vref is the total volume of above brain regions.

RT-PCR and in situ hybridization assay. Total RNA was extracted from the brain of *T. pavo* ($n=3$) using TRI reagent (Sigma, Italy) and dissolved in DEPC-water (Sigma, Milan, Italy). The reverse transcription reaction was performed using 2 μg of total RNA with RETRO-script kit (Celbio, Milan, Italy) at 44 °C for 1 h after template denaturation at 75 °C for 3 min. Polymerase chain reaction (PCR) was handled for HSP70 and for HSP90 gene using Taq Polymerase (Promega, Italy) with degenerate primers: HSP70 (sense 5′ACCGTGCCC-GCCTAYTTYA; antisense 5′ACGACAGCGTCCTYTTNGC); HSP90 (sense

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