C. R. Biologies xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Comptes Rendus Biologies

www.sciencedirect.com

Molecular biology and genetics/Biologie et génétique moléculaires

Retinoblastoma binding protein 6 and crystallin lambda 1 are cadmium-responsive genes in zebrafish embryos and adults retinae

Rosaria Scudiero [a,*], Maria Grazia Esposito [a], Palma Simoniello [b], Chiara Maria Motta [a]

[a] Department of Biology, University of Naples Federico II, via Mezzocannone 8, 80134 Naples, Italy
[b] Department of Sciences and Technology, University of Naples Parthenope, Naples, Italy

ARTICLE INFO

Article history:
Received 3 January 2017
Accepted after revision 8 March 2017
Available online xxx

Keywords:
Cadmium
Gene expression
In situ hybridization
mRNA differential display
Zebrafish embryos
Adult zebrafish retina

ABSTRACT

Nonessential metal cadmium is widely used and released in the environment, causing cell toxicity and posing a severe threat to wildlife. Zebrafish (*Danio rerio*) is one of the most commonly used animals in the investigation of environmental cadmium toxicity in vertebrates. In this study, we identified two cadmium-responsive genes, RBBP6 and CRYL1, in the early phases of zebrafish development, at the gastrula stage. The retinoblastoma binding protein 6 is associated with increased protein degradation and cell proliferation; crystallin-lambda 1 is a lens protein with redox activity. In situ hybridization analysis performed on adult zebrafish exposed to 1.5–40 μM cadmium for 30 days confirmed the ability of cadmium to up-regulate the expression of both genes in retinal cells in a dose-dependent manner. The over-expression was transient, being switched off when cadmium was removed. The involvement of RBBP6 and CRYL1 in the onset of cadmium-induced morphological alterations in adult zebrafish retina is discussed.

1. Introduction

The marked toxicity of cadmium (Cd) ions on aquatic organisms with respect to the terrestrial counterparts is commonly attributed to their higher bioavailability in water [1] and to the multiple routes of uptake (skin, gills, gut) [2,3]. In both marine and freshwater habitats, Cd exerts its toxicity even at traces environmental concentrations, causing severe damage at the morphocytological level. Cd, for example, impairs tissue organization in the skeletal muscles [4,5], in the gills, liver and kidney [6–8], in the neuroglia in the central nervous system [9] and in the retina [10,11].

Cd exposure also alters gene expression [11–15] but the correlation existing between the observed molecular and morphological changes remains up to now rather unclear [16].

With the aim to identify early genes involved in cellular response to Cd stress in vertebrate embryos, we started a transcriptomic study using zebrafish as model organisms. The preliminary results herein described demonstrated a Cd-induction of the expression of the retinoblastoma binding protein 6 (*RBBP6*) and the crystallin lambda 1 protein (*CRYL1*) genes in gastrula embryos. RBBP6 protein is an E3 ubiquitin ligase involved in protein degradation [17]; the protein interacts with both p53 and pRb, promoting their degradation and thereby increasing cell

* Corresponding author.
E-mail address: rosaria.scudiero@unina.it (R. Scudiero).

http://dx.doi.org/10.1016/j.crvi.2017.03.004
1631-0691/

proliferation. CRYL1 is a lens protein with enzymatic features first identified in rabbit and hare [18], down-regulated in hepatic cancer cells [19,20]. These two genes are both involved in eye development and in cellular response to cell diseases [21–23]. Since zebrafish eyes are particularly sensitive to Cd toxicity [10,11,24], we decided to investigate the expression of these 2 genes in zebrafish adult eyes by using *in situ* hybridization analysis, in natural conditions and after 30 days of Cd-exposure. In particular, an environmental concentration [5,11] and two sub-lethal concentrations (20 and 40 μM) were tested. To obtain information on the reversibility of the treatment, a group of animals exposed to 40 μM were maintained for 7 days in clean water and then subjected to *in situ* hybridization. The analyses demonstrated that both λ-crystallin and retinoblastoma binding protein 6 are Cd-sensitive and are upregulated by the metal in a dose-dependent manner.

Our data suggest an involvement of RBBP6 and CRYL1 proteins in the onset of morphological alterations previously observed in the zebrafish retina after exposure to cadmium.

2. Materials and methods

2.1. Animals and cadmium treatments

Adult and healthy zebrafish (*Danio rerio*) of both sexes were obtained from a specialized supplier and kept in standard conditions as previously described [25] in a 50-L housing tank for at least 2 weeks to acclimate before the experiments. Embryos were obtained as described by Westerfield [26]. Animal collection and housing were approved by the National Committee of the Italian ministry of health, and all experiments were conducted with the authority of the University Federico II ethical animal care and use committee. The experiments complied with the current laws of the European Union.

At the developmental stages of blastula (4 h after fertilization), an experimental group of 100 embryos were allowed to grow for 4 h in the embryo's medium added with $CdCl_2$ for a final concentration of 20 μM Cd. Progression of embryonic development was scored by light microscopic observation. At the end of the treatment, embryos at the developmental stages of gastrula (approximately 80% epiboly) were washed, collected and processed for molecular analysis.

For the treatment of adult specimens, the animals were separated in four groups of 20 and housed in 10-L tanks with the respective treatment. The animals were transferred to the test aquarium filled with reverse osmosis water and kept in continuously aerated water, 25 ± 2 C, under a 14–10 h light/dark cycle photoperiod, fed three times a day with TetraMin Tropical Flake fish. The treatments were as follows: group 1, 1.5 μM Cd; group 2, 20 μM Cd; group 3, 40 μM Cd. The control group (group 4) was kept in the same conditions as the other groups, but without the addition of cadmium in the reverse osmosis water. Treatment was static (solutions remained unchanged throughout the duration of the test), lasted for 30 days and did not induce any evidence of physical or behavioral stress.

2.2. Total RNA isolation

Total RNAs from zebrafish embryos developed under natural (control) and Cd-contaminated (Cd-treated) conditions were extracted according to the TRI-REAGENT (Sigma Aldrich) protocol. Turbo DNA-*free* kit (Ambion) was used to digest the trace amounts of genomic DNA contamination in RNA. The two populations of total RNAs were dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −75 °C. The concentration and purity of RNA samples were determined by UV absorbance spectrophotometry; RNA integrity was checked using formaldehyde–agarose gel electrophoresis.

2.3. Differential display reverse transcriptase (RT)-PCR

DDRT-PCR was essentially performed as described previously [12,27]. Briefly, DNA-free total RNA (0.4 μg) extracted from either control or Cd-treated zebrafish embryos was reverse-transcribed in a 20-μL reaction mixture at 37 °C for 60 min with MMLV-reverse transcriptase and a set of two, one base anchored oligo(dT) primers. Amplification of cDNA fragments was performed using combinations of the anchored primers from the reverse-transcription step and 10 different upstream primers; all PCRs were repeated twice using the same cDNA sample. Aliquots (3 μL each) of amplification products were resolved on a 2% agarose gel (20 × 25 cm) with ethidium bromide in TAE buffer. Using a sharp, clean razor blade, a rectangular piece of gel corresponding to an individual band of interest was excised and the cDNA fragment recovered using a gel extraction kit (5PRIME). The eluted cDNA was re-amplified in a PCR reaction using the same pair of primers used in the differential display reaction. The Cd-responsive fragments were inserted into a pCRII-TOPO vector (Invitrogen) and cloned following the manufacturer's instructions. Recombinant plasmid DNA was sequenced bidirectionally by the DNA sequencing service of Primm Biotech, using T7 and T3 primers. The homology search of genes was performed by online-based FASTA and BLAST programs through the European Molecular Biology Laboratory (EMBL) nucleotide sequence database at European Bioinformatics Institute (http://www.ebi.ac.uk).

2.4. In situ hybridization

At the end of the treatments, adult zebrafish were anesthetized with MS222 (tricaine methanesulfonate, 1:15,000 w/v) and sacrificed by decapitation. Heads were rapidly dissected, fixed in Bouin's solution and processed for paraffin wax embedding according to routine protocols [28]. Sections (5–7 μm) were mounted on superfrost glass slides (Menzel-Glaser, Germany) and used for *in situ* hybridization [29]. In particular, they were fixed in paraformaldehyde 4% PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) pH 7.4 for 20 min, and incubated in PK buffer (Tris-HCl, 0.2 M, pH 7.4, EDTA 0.01 M, pH 8, proteinase K, 10 mg/mL, H_2O_{depc}) at 37 °C for 15 min. After washing in PBS, they were incubated at 42 °C for 90 min in a prehybridization mix containing formamide, SSC 4× and 1× Denhart's solution. Hybridization was

Please cite this article in press as: R. Scudiero, et al., Retinoblastoma binding protein 6 and crystallin lambda 1 are cadmium-responsive genes in zebrafish embryos and adults retinae, C. R. Biologies (2017), http://dx.doi.org/10.1016/j.crvi.2017.03.004