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Effects of Aroclor 1254 on oxidative stress in developing *Xenopus laevis* tadpoles ☆

Virginie Gillardin [a,*], Frédéric Silvestre [a], Céline Divoy [a], Jean-Pierre Thomé [b], Patrick Kestemont [a]

[a] Unité de Recherche en Biologie des Organismes (URBO), Facultés Universitaires Notre-Dame de la Paix, Rue de Bruxelles 61, B-5000 Namur, Belgium
[b] Laboratoire d'Ecologie Animale et Ecotoxicologie (LEAE), Université de Liège, Belgium

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ABSTRACT

Over the last decades, amphibians decline has been reported worldwide. Exposure to polychlorinated biphenyls (PCBs) is one of the possible causes in addition to climate changes, UV-radiation or habitat destruction. In the present study, we tested the hypothesis that PCBs could induce oxidative stress in young tadpoles. Developing *Xenopus laevis* were exposed from 2- to 5-d postfertilization (pf) to 0.1 or 1 mg/l of Aroclor 1254. Lipid peroxidation and antioxidant systems (SOD, CAT, GST, GPx, GR activities and t-GSH level) were investigated in whole organisms. Exposure to both concentrations did not impact on the survival and development whereas the average body weight decreased. Exposure to 1 mg/l of Aroclor 1254 induced a significant ($p<0.05$) increase of GST activity when compared to controls 0 and DMSO. The other antioxidant enzymes and LPO evaluation remained unchanged. Our results demonstrate that exposure of *X. laevis* tadpoles to environmental concentrations of Aroclor 1254 interfere with normal growth. They also highlight that very young *X. laevis* tadpoles express antioxidant systems.

1. Introduction

Over the last decades, many amphibian populations declined in a number of geographical locations worldwide (Stuart et al., 2004; Blaustein and Dobson, 2006; Pasmans et al., 2006). In most reported cases, causes are assumed to result from man-made alterations to the environment such as habitat destruction or UV-radiation (Carey and Bryant, 1995). However, exposure to environmental pollutants like polychlorinated biphenyls (PCBs) should now be taken into account (Glennemeier and Denver, 2001).

PCBs are persistent, widely distributed environmental contaminants. They were manufactured in the 1950s for use in electrical insulators, plasticizers and carbonless copy paper (Safe, 1994). Twenty years after their production ban in most industrialized countries, PCBs are still present in the environment (Ulbrich and Stahlmann, 2004).

Potential toxic effects of PCBs have been investigated in numerous species and among them, amphibians are newly becoming centre of interest. It has been reported that PCBs induce mortality (Savage et al., 2002), developmental deformities (Gutleb et al., 1999, 2000; Jelaso et al., 2002; Fisher et al., 2003), delays in metamorphosis (Lehigh-Shirey et al., 2006), immunological effects (Linzey et al., 2003), disruption of gonads development (Qin et al., 2003, 2005) and changes in gene expression (Jelaso et al., 2002, 2003, 2005) in developing amphibians.

Oxidative stress has been postulated to play a role in the toxic manifestations of chlorinated compounds such as tetrachlorodibenzo-*p*-dioxin (TCDD) and PCBs. Indeed, TCDD and PCBs induce oxidative stress in rat (Twaroski et al., 2001; Fadhel et al., 2002; Venkataraman et al., 2004; Muthuvel et al., 2006), bird (Hoffman et al., 1996; Hilscherova et al., 2003) and fish (Orbea et al., 2002; Ruiz-Leal and George, 2004; Vega-Lopez et al., 2006). Moreover, PCBs are responsible for oxidative stress status and teratologic effects in chick embryos (Jin et al., 2001; Katynski et al., 2004).

It is usually admitted that PCBs are associated with the activation of the cytosolic aryl hydrocarbon receptor (AHR), which is followed by increased expression and activity of cytochrome P4501A (CYP1A) and other phase I and phase II enzymes. Production of reactive oxygen species (ROS) could result from a wide variety of AHR-associated mechanisms, including altered expression and activity of CYP1A (Safe, 2001; Dalton et al., 2002). Various enzyme systems such as cyclooxygenase (COX), nitric oxide synthase (NOS), xanthine oxidase, ribonucleotide reductase,

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* Corresponding author. Fax: +32 81 72 43 62.
E-mail addresses: virginie.gillardin@fundp.ac.be, vgillard@fundp.ac.be (V. Gillardin).

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mitochondrial electron transport systems, and NADPH oxidase are also involved in the production of ROS in response to various stimuli (Curtin et al., 2002).

Production of ROS can induce formation of hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) which can lead to extensive damage to proteins, lipids and nucleic acids. Cells contain enzymatic and nonenzymatic antioxidant defense systems for ROS detoxification like superoxide dismutases (SODs), catalase (CAT), glutathione redox cycle enzymes, carotenoids, vitamin A and E, etc. (Kashiwagi et al., 2005). Normal homoeostasis status requires a balance between prooxidant and antioxidant systems (Hilscherova et al., 2003). Exceeding the defensive capability of antioxidant systems can induce adverse effects.

So far, no study has focused on the potential action of PCBs as oxidative stress inducer in developing amphibians. Therefore, this study investigated the possible activation of antioxidant systems in developing *Xenopus laevis* tadpoles exposed to environmental concentration of the PCBs mixture Aroclor 1254 (0.1 and 1 mg/l). In Europe, Covaci et al. (2005) have reported concentrations of PCBs around 0.4 μg/g in the sediment of the river Scheldt (Belgium) and previous investigations of the Belgian ponds fauna by our research group indicated concentrations of PCBs (Σ 24 congeners) between 0.001 and 2 μg/g lipid weight in snail tissues (unpublished data). Routine markers of toxicity were assessed (mortality, PCBs burdens in tadpoles, final average body weight) and activity of glutathione *S*-transferase (GST) phase II enzyme was examined. Total glutathione (t-GSH) level and lipid peroxidation were also investigated. Finally, antioxidant enzymes (SOD, CAT, glutathione redox cycle enzymes) activities were characterized in order to highlight a potential activation of the enzymatic antioxidant defense system.

2. Material and methods

2.1. Animals, breeding and housing

Adult African clawed frogs *X. laevis* were obtained in 2004 from the National Breeding of *Xenopus*, University of Rennes, France. Animals were maintained in glass container in dechlorinated water at 22±1 °C with a 12:12 photoperiod schedule. Fresh water was exchanged every 2 days. Animals were fed thrice weekly with commercial trout food (Trouw France, Fontaine les Vervins, France) or chironomid larvae. Breeding was induced by subcutaneous injection of adults with 750 IU of human chorionic gonadotropin hCG (Sigma, Steinheim, Germany). Cleaving embryos (stages 8–13 of Nieuwkoop and Faber, 1994) were placed in FETAX medium (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$ and 75 mg $SO_4 \cdot 7H_2O$ per l distilled water) until they hatched (48 h postfertilization (pf)).

2.2. Chemical exposure

Normally developing tadpoles (stage 35/36) were placed in glass bowls filled with 200 ml of FETAX medium. Each experimental condition included three replicates of 20–25 tadpoles. The PCBs mixture Aroclor 1254 (ALLTECH Associates Inc., Lokeren, Belgium) was added to medium using dimethylsulphoxide (DMSO) (Sigma) (final concentration of 0.05%) as a solvent, resulting in nominal concentrations of 0.1 and 1 mg/l. A medium control group and a DMSO solvent (0.05%) control group were included in each experiment. During the assay, the temperature was maintained at 22±1 °C, the solutions were changed every 24 h and dead tadpoles were removed daily. When tadpoles reached stage 45 (4-d pf), they were fed a mixture of spirulin algae (Sera, Heinberg, Germany). After 76 h of exposure, survival rate and developmental stages were recorded. Surviving tadpoles of each bowls were pooled and weighed, snapfrozen in liquid nitrogen and stored at −80 °C. For each treatment, one replicate was assigned for biochemical and another for chemical analysis. The third replicate was kept to further molecular analysis. Each experiment was repeated four times with tadpoles obtained from independent spawnings.

2.3. Sample preparation

Pooled tadpoles were homogenized 1:5 w:v in ice-cold 50 mM phosphate buffer (pH 7.5) containing 0.25% protease inhibitor cocktail (Sigma). Two hundreds microliters of homogenate were taken for both lipid peroxidation and reduced glutathione assay. The rest of the homogenate was centrifuged at 1000*g* for 10 min, aliquoted into portions and kept at −80 °C prior to biochemical analysis.

2.4. Enzyme activities, t-GSH and lipid peroxidation

Total SOD activity was measured using the method of Mockett et al. (2002) based on the production of $O_2^{\bullet -}$ by xanthine–xanthine oxidase, which reduces nitroblue tetrazolium (NBT) to a blue formazan. Inhibition of the reaction by SOD was monitored spectrophotometrically at 560 nm for 6 min. CAT activity was estimated spectrophotometrically by the titanium oxysulphate ($TiOSO_4$) method (Baudhuin et al., 1964) with some modifications. Glutathione peroxidase (GPx) activity was determined using the method of Flohe and Günzler (1984) as modified by Mohandas et al. (1984) in which the oxidation of NADPH was recorded spectrophotometrically at 340 nm at 37 °C. Glutathion reductase (GR) activity was measured by the method of Carlberg and Mannervik (1975) as modified by Mohandas et al. (1984). The enzyme activity was quantified at 30 °C by measuring the disappearance of NADPH at 340 nm. GST activity was determined using the method of Habig et al. (1974). The conjugation of GSH with CDNB via GST activity at 25 °C was recorded spectrophotometrically at 340 nm.

The t-GSH content was estimated using the method of Baker et al. (1990). Reaction of reduced GSH with DTNB was measured spectrophotometrically at 412 nm.

Lipid peroxidation that occurs with free radical generation and results in the production of malondialdehyde (MDA) was assessed by the thiobarbituric acid (TBA) method (Fatima et al., 2000). MDA react with TBA and the product is read spectrophotometrically at 535 nm.

Protein contents in all samples were assayed by the method of Lowry et al. (1951) using Folin's reagent and BSA as standard.

2.5. PCBs analysis

PCBs were extracted according to a slight modification of EPA method 608 as previously described by Debier et al. (2003) with modifications for analysis with tadpoles. Twenty-four PCB congeners (from di- to nonachlorinated) (IUPAC nos. 28, 44, 52, 66, 70, 87, 95, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194, 195, 206, 209) were identified and quantified. Among these 24 PCB congeners, the concentration of 17 pure PCB congeners (IUPAC nos. 44, 52, 66, 70, 77, 95, 101, 110, 118, 128, 138, 149, 156, 170, 180, 183, 187) was calculated from the added amount of Mirex used as internal standard. This set of 17 pure components represent 55.71% of Aroclor 1254 by weight. The total PCB concentration, expressed in Aroclor 1254 equivalents, was calculated by means of the formula developed by Thomé et al. (1995). PCBs concentrations were transformed in Aroclor 1254 equivalent and expressed in μg/g lipids and in μg/g body weight. All the tadpoles assigned for chemical analysis were pooled to obtain about 100 mg of lipid after extraction. Extraction of lipids was performed with hexane using an accelerated solvent extractor (ASE) (Dionex 200, Sunnyvale, USA). All the extracts were used for lipids contents determination: solvent was evaporated using a Turbovap LV (Zymarck, Hopkinton, MA, USA) until a constant weight was obtained. Samples were then diluted in 3 ml *n*-hexane and a surrogate (PCB 112 with a final concentration of 50 pg/μl) was added in order to quantify possible loss of PCBs during the procedure. The extracts were subjected to cleanup with sulphuric acid in order to remove organic matter (lipids, lipoproteins, glucides), 2 ml of a mixture of concentrate (95%) and fuming (30%) sulphuric acid (3:1; v:v) were added to the extract. The mixture was shaken and centrifugated at 1750*g*. The supernatant was removed and 3 ml *n*-hexane were added to the decanted acid. Shaking, centrifugation and removal were performed a second time before evaporation of the solvent. A cleanup column (Superclean™ ENVI Florisil SPE tubes 6 ml, SUPELCO, Bellefonte, USA) was also used in order to remove polar molecules. Columns were eluted with 5 ml acetone, 5 ml acetone–hexane and 12 ml hexane before the extracts were eluted with 6 ml hexane. After the addition of 125 μl of a surrogate (PCB30, 100 pg/μl diluted in hexane) and 125 μl of an internal standard (Mirex 100 pg/μl diluted in hexane), the extracts were analysed using a high-resolution gas chromatograph (Thermoquest, Trace 2000, Milano, Italy) equipped with a ^{63}Ni electron capture detector (ECD-^{63}Ni). PCBs were separated by progressive temperature increase. Congeners were finally identified and quantified according to their retention time thanks to the software Chrom-Card for Windows 4.0. The quantification limit of PCBs in tissue was 1 ng/g w:w and 200 ng/g lipids.

2.6. Statistical analysis

Results were expressed as the mean ($n = 4$)±standard deviation of the mean SD. Normality analysis of data was assessed by Kolmogorov–Smirnov test. Homogeneity of variances was tested by Bartlett test. Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Post-hoc LSD multiple comparison test at a 5% significant level. All statistical analysis were performed using Statistica™ software for Windows (StatSoft, Tulsa, OK, USA).