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Identification of genes expressed as a result of lindane exposure in *Oreochromis niloticus* using differential display

Reyna Colli-Dulá [a], José-Juan Zúñiga-Aguilar [b], Arnulfo Albores-Medina [c], Omar Zapata-Perez [a,*]

[a] Departamento de Recursos del Mar, Cinvestav Unidad Merida, Km 6 Antigua Carretera a Progreso, Col. Cordemex. C.P. 97300, Merida, Yucatan, Mexico
[b] Unidad de Bioquimica y Biologia Molecular de Plantas, CICY, México
[c] Sección Externa de Toxicología, Cinvestav Zacatenco. Mexico D.F., Mexico

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ABSTRACT

In order to assess the effect of lindane exposure on gene expression in tilapia (*Oreochromis niloticus*), twenty male fish were individually weighted and injected intraperitoneally with a single dose of lindane (19.09 mg/kg bw) using corn oil as a carrier vehicle, while a second group of twenty male fish (controls) was only injected with the carrier vehicle. Groups of four fish each were then sacrificed at 3, 6, 12, 18 and 24 h after treatment application and total RNA was extracted from liver tissue. The differential display (DD) technique was then used to identify differentially expressed cDNA fragments between treatment and control fish. A total of fifty cDNA fragments were isolated and sequenced, from which only four showed homology with genes previously described in other fish species, namely the immunoglobulin heavy chain (IgH), coagulation factor V (FV), casein kinase 2 alpha (CK2a), and the receptor protein-tyrosine-like phosphatase (RPT-LP). The expression of such genes was confirmed using quantitative real time-polymerase chain reaction (QRT-PCR). Results showed that lindane exposure triggered the differential expression of these genes during the first 6, 18 and 24 h subsequent to treatment application, suggesting that lindane exposure can trigger a rapid immune system response in tilapias.

1. Introduction

Several organochlorine pesticides such as lindane (γ-hexachlorocyclohexane (HCH)) have been widely used in agriculture to protect crops from herbivorous insects, as well as to control insect-borne diseases that are relevant to human health and veterinary medicine (Worthing, 1987; US EPA, 1993). Although their use has been progressively banned worldwide (Weinhold, 2001; US EPA, 2002), they are still commonly used in countries such as Mexico, Guatemala, Nicaragua, Salvador, and in several African nations. Numerous studies with fish have shown that a chronic exposure to lindane may cause physiological effects, which include negative effects on development and reproduction, behavioral changes (Ensenbach and Nagel, 1997; Hanson et al., 2007), changes in the lipid metabolism (Singh, 1992; Singh et al., 1994), altered enzymatic activity (Ferrando et al., 1991, 1992; Betoulle et al., 2000), as well as changes in the immune and endocrine systems (Cossarini-Dunier et al., 1987; Hart et al.,1997; Arukwe et al., 2000; Singh and Canario, 2004). Furthermore, studies with yeasts have also shown that lindane elicits the differential expression of genes involved in mitochondrial functioning, organization and biogenesis, oxidative stress, and ionic homeostasis (Parveen et al., 2003).

The detection of changes in gene expression has become a highly sensitive and faithful bioassay to evaluate biochemical and physiological effects caused by environmental pollutants (Larkin et al., 2002; Lemaire et al., 2005; Tanguy et al., 2005; Garcia-Reyero et al., 2006; Griffitt et al., 2006; Lee et al., 2006; Filby et al., 2007). Nonetheless, most studies have focused on the physiological effects of pesticides such as lindane in fish species, and there is virtually no information on changes in gene regulation in response to lindane exposure or lindane detoxification and/or biotransformation mechanisms.

Differential display reverse transcription polymerase chain reaction (DDRT-PCR) is one of the most commonly used molecular techniques to detect and characterize differential gene expression in eukaryotic cell populations (Liang and Pardee, 1992; Liang, 2002, 2006). This method has been used to simultaneously identify differentially expressed genes across multiple samples, and due to its sensitivity and high specificity, it does not require any prior knowledge on sequences. It also allows for the direct identification of changes in mRNA levels. Traditionally, this technique has been primarily used to isolate genes involved in physiological processes, signal transduction, stress response and

* Corresponding author. Fax: +52 999 9 81 23 34.
E-mail address: ozapata@mda.cinvestav.mx (O. Zapata-Perez).

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secondary metabolism, and not until recently it started being used for early detection of exposure to contaminants in fishes (Denslow et al., 2001; Bain, 2002; Peterson and Bain, 2004; Roling et al., 2004; Yoo et al., 2007). In this way, we consider our study provides a relevant contribution as most studies that have focused on the effects of lindane have focused their discussion almost exclusively on human health or vertebrate wildlife toxicity.

2. Materials and methods

2.1. Chemicals

Lindane (technical grade 99.55% purity) was obtained from Sigma-Aldrich (Mexico), and used with no further purification: γ-isomer of hexachlorocyclohexane. TRI-Reagent was also purchased from Sigma and RNase-free DNase I from Promega (Madison, WI, USA).

2.2. Fish culture and treatments

Forty juvenile male tilapias between 50 and 75 g of weight and 14–17 cm in total length were carefully collected from CINVESTAV-Merida farms. Fish were maintained at 25±3 °C in 500l tanks with aerated water, under natural photoperiod and flow-through conditions during a 4-week acclimation period.

2.3. Exposure of male tilapias with lindane

Twenty male fish were then randomly chosen, individually weighted and injected intraperitoneally with a single dose of lindane. A stock solution of lindane with 9.54 mg/mL dissolved in corn oil as carrier vehicle was used to give final concentrations of 19.09 mg/kg of body weight. Additionally, another 20 fish were also weighted and then injected only with corn oil, and served as a control group. After treatment application, four fish from each treatment group were randomly drawn at five different time intervals (3, 6, 12, 18 and 24 h). In order to guarantee the low-effect adverse level (LOEAL), the mentioned dose of lindane was selected based on the results of preliminary experiments performed in our lab and based on previous studies of physiological responses in fish (Dunier and Siwicki, 1994; Geyer et al., 1994; Siwicki and Dunier, 1994; Hart et al., 1997; Hirthe et al., 2001; Singh and Canario, 2004). The selected specimens were sacrificed and their livers were extracted and placed in tubes and stored at −80 °C for molecular analyses. Specimen mortality was not observed throughout the course of the experiment. All animal experiments were conducted in accordance with the Guidelines for Care and Manipulation of Laboratory Animals (PICUAL-Cinvestav, 2002), the Mexican Official Norm (de Aluja, 2002, NOM-062-ZOO-1999) and the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of Health.

2.4. RNA isolation

Following the manufacturer's instructions, total RNA was extracted from a 0.5 g liver sample using TRI-Reagent (Sigma). Total RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove residual genomic DNA. The RNA concentration was estimated based on absorbance at 260 nm using a BioMate 3 Spectrophotometer (Thermo Electron Corporation, Madison, WI, USA); RNA quality was verified by electrophoresis using agarose gels (1.5%) stained with ethidium bromide, and by ensuring 260/280-nm OD ratios between 1.8 and 2.0.

2.5. Differential display RT-PCR

The differential display procedure was performed with pooled mRNA samples ($n=4$) belonging to each time interval (3, 6, 12, 18 and 24 h after injection) for both control and lindane-treated groups using the Delta® Differential Display Kit (CLONTECH Laboratories, Inc.) and following the manufacturer's instructions. Total DNA-free RNA (5 μg) was heat-denaturalized and reverse-transcribed at 42 °C for 60 min in 25 μL of reaction buffer (50 mM Tris–HCl, pH 8.3, 2 mM $MgCl_2$), 0.4 mM of dNTP mixture, 20 ng/μL of oligo$(dT)_{12-18}$ and 200 units of MMLV-reverse transcriptase. The reaction mix was incubated at 75 °C for 15 min to achieve enzyme inactivation, and then placed on ice. PCR was carried out with 2 μL of single-strand cDNA aliquots using seven different combinations of downstream (T) and upstream (P) primers (T1:P1, T2:P1, T3:P1, T4:P1, T8:P8, T1:P8 and T2:P8, from the Clontech Deltas Differential Display Kit) in 50 μl of PCR buffer 10X, 50 mM of $MgCl_2$, 10 mM of dNTPs, 20 ng/μL of each P and T primer, and a 0.25 unit of Taq DNA polymerase (Invitrogen). Reaction conditions were programmed as follows: 1 cycle at 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min; 2 cycles at 94 °C for 5 min, 40 °C for 5 min, 72 °C for 5 min; 38 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 5 min, and a final elongation step at 72 °C for 7 min. In order to compare product patterns, 5 μl of PCR products were fractionated in denaturalizing 6% polyacrylamide/7 M urea sequencing gels in a 0.5X TBE buffer, at 70 W for 3.5 h. After electrophoresis, polyacrylamide gels were glass-fixed and silver-stained using the protocol described in Bassam et al. (1991). Differentially expressed cDNA bands were excised from dried gels and incubated in 40 μl of TE buffer at 100 °C for 5 min. The eluted cDNAs were re-amplified using the original PCR conditions and primer combination set.

2.6. Cloning and sequencing of differential display products

PCR products were ligated into the pGEM-T Easy Vector System 1 (Promega, Madison, WI) and used to transform *E. Coli*-competent cells using standard cloning protocols (Sambrook and Russell, 2001). Plasmids were extracted from white colonies using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced at the National Laboratory of Genomics in CINVESTAV-Irapuato (Mexico City). cDNA sequences were compared in the NCBI GenBank database using Blast (http://www.ncbi.nlm.nih.gov/BLAST/).

2.7. Quantitative real time-PCR (QRT-PCR)

Genes that exhibited differential expression were confirmed by quantitative real time-PCR (QRT-PCR), and their sequences were designed according to requirements in Primer Express v3.0. Primer sequences are shown in Table 1. PCR amplifications were performed in a total volume of 25 μL which contained 10 μM of each primer, 12.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 9.5 μL of dH_2O, and 2.5 ng of cDNA template (2 μL of 1.5 ng/μL). All QRT-PCR assays were triplicate and carried out with an ABI PRISM 7500 Real-Time PCR System using the following cycle conditions: 10 min at 95 °C, followed by 45 cycles of PCR amplifications (melting during 20 s at 95 °C, anneal and extend 1 min at 58 °C, and then a dissociation curve calculation step). Annealing temperatures for each primer are given in Table 1. QRT-PCR data were analyzed using the ABI PRISM 7500 Sequence Detection System Software, ver. 1.3.1 (Applied Biosystems). The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence level rises significantly above the background level. To obtain quantitative PCR data from the differential expression analysis, PCR efficiencies of target and reference genes were validated with homology. The $dd\boldsymbol{C}_T$ calculation ($dd\boldsymbol{C}_T = dCt^{Target} - dCt^{Reference}$) for relative quantification of targeted genes was employed, using β-Actin (Ambion) as the universal reference gene. Real-time PCR data were analyzed using the 2^{ddC_T} method (Livak and Schmittgen, 2001) where $dd\boldsymbol{C}_T = (dCt^{\text{Treatment target}} - dCt^{\beta\text{-Actin}}) - (dCt^{\text{Control target}} - dCt^{\beta\text{-Actin}})$, and 2^{ddC_T} = fold change.

2.8. Statistical analyses

Results are reported as medians±interquantile range. All statistical tests were nonparametric. Significant differences between genes expressed in control and lindane-treated groups for each time interval were evaluated by means of a Kruskal–Wallis tests (significance level set at 0.05). Statistical analyses were carried out using Statistica, version 5.5 (Statsoft, Tulsa, OK).

Table 1
Sequences of primers used in the QRT-PCR analysis.

Clone name	Forward primer sequence 5′→3′	Reverse primer sequence 5′→3′	Annealing temperature (°C)
DD1	CCGCACTCAATGAGCTGAATAC	TGCATTACAGTCCCTGGACACT	65.5
DD3	AGGATGTACACCAACCAGCTTGT	CCATGAAAGTGGACACTATGAATATCA	65.5
DD5	TGACAAACTGCTGCGTTACGA	GGGATAGAAGTAAGGATGATCCAT	66.5
DD9	GGTCAACATCATGCCCTACGA	CTGATTGGCTGCAGACAGACA	58.0