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Differential gene expression in rainbow trout (*Oncorhynchus mykiss*) liver and ovary after exposure to zearalenone

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ABSTRACT

Zearalenone (ZEA) is a mycotoxin of worldwide occurrence, and it has been shown to produce numerous adverse effects in both laboratory and domestic animals. However, regardless of recent achievements, the molecular mechanisms underlying ZEA toxicity remain elusive, and little is known about transcriptome changes of fish cells in response to ZEA occurrence. In the present study, differential display PCR was used to generate a unique cDNA fingerprint of differentially expressed transcripts in the liver and ovary of juvenile rainbow trout after either 24, 72, or 168 h of intraperitoneal exposure to ZEA (10 mg/kg of body mass). From a total of 59 isolated cDNA bands (ESTs), 5 could be confirmed with Real-Time qPCR and their nucleotide sequences were identified as mRNAs of: *acty* (β-centractin), the cytoskeleton structural element; *bccip*, responsible for DNA repair and cell cycle control; *enoa* (α-enolase), encoding enzyme of the glycolysis process; *proc* (protein C), that takes part in the blood coagulation process; and *frih*, encoding the heavy chain of ferritin, the protein complex important for iron storage. Further qPCR analysis of the confirmed ESTs expression profiles revealed significant mRNA level alterations in both tissues of exposed fish during the 168 h study. The results revealed a complex network of genes associated with different biological processes that may be engaged in the cellular response to ZEA exposure, i.e. blood coagulation or iron-storage processes.

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1. Introduction

Zearalenone (ZEA) is a mycotoxin produced mainly by various fungi of the *Fusarium* genus, which are frequently found in cereals and other plant products, resulting in worldwide contamination of food and animal feeds (Zinedine et al., 2007; Fink-Gremmels, 2008). The chemical structure of ZEA (described as 6-[10-hydroxy-6-oxo-*E*-1-undecenyl]-β-resorcyclic acid lactone) is stable, making this mycotoxin resistant to postprocessing methods, i.e. storage, milling, food processing, and cooking (Lauren and Smith, 2001). Despite the hydrophobic character of the molecule, ZEA was also found in surface and ground waters (Laganà et al., 2004; Gromadzka et al., 2009) which suggests a possible environmental threat for aquatic animals, including fish.

Numerous toxicological and epidemiological studies have revealed an array of negative health effects in animals exposed to ZEA, however the molecular background of ZEA exposure is still not fully understood. The most widely acknowledged feature of ZEA is its ability to bind to estrogen receptor (ER) and induce expression of estrogen responsive genes (e.g. Olsen et al., 2005; Takemura et al., 2007). Consistent with that potential, exposure to ZEA leads to a number of reproductive disorders in mammals, e.g. decreased libido, anovulation, infertility, or neoplasmic lesions, which all derive from the mechanisms that alter transcription of ER-dependent genes (Tiemann et al., 2003; Zinedine et al., 2007; Minervini and Aquila, 2008; Jakimiuk et al., 2009). The few in vivo studies on fish yielded similar results: ZEA and its metabolites modulated expression of ER-responsive genes, and affected the immune system, growth, and reproduction (Arukwe et al., 1999; Keles et al., 2002; Woźny et al., 2008, 2010; Schwartz et al., 2010). Moreover, recent achievements suggest that ZEA is capable of interfering with other receptor systems and signaling cascades that are known from cross-talk activity with the ER pathway, i.e. aryl hydrocarbon receptor, constitutive androstane receptor, or pregnane X receptor (Ding et al., 2006; Ayed-Boussema et al., 2011).

While most of the biological properties of ZEA are believed to be attributed to the agonistic effect on the ER, certain biological responses to this mycotoxin cannot be simply explained by its estrogenic activity. For example, in exposed laboratory rodents, ZEA induced liver injury and impairment of the blood coagulation process (Maaroufi et al., 1996; Abbès et al., 2006). Other reports show that ZEA is cyto- and genotoxic as it exhibits the potential in vitro and in vivo to induce micronuclei, chromosome aberrations, DNA fragmentation, cell cycle arrest, and apoptosis (Ouanes et al., 2005; Ayed-Boussema et al., 2007, 2008), which

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all constitute responses to oxidative injury. In fact, ZEA has recently been classified as a highly oxidant mycotoxin (El Golli-Bennour and Bacha, 2011), suggesting that oxidative damage may be the predominant toxic effect for ZEA action. However, it is important to bear in mind that the detrimental effects of ZEA, linked to oxidative stress of animal organs, were observed mostly in studies using environmentally non-relevant ZEA concentrations (Maaroufi et al., 1996; Abbès et al., 2006).

Based on the findings summarized above, it is almost certain that the molecular mechanism of ZEA action involves a complex web of altered gene expression. Identification of differentially expressed genes becomes a major undertaking in the evaluation of these changes which may be important in determining the final outcome of ZEA toxicity. Modern tools of molecular biology, like microarrays and DNA chips, have recently opened a new paragraph in the field of biomarker selection and pathway analysis. However, differential display PCR (DD-PCR), a method of choice that has been used for almost two decades (Sokolov and Prockop, 1994), still offers a robust alternative for performing transcriptome analysis at low cost (e.g. Brzuzan et al., 2007; Luparello et al., 2012).

The aim of this research was to investigate in vivo effects of ZEA exposure on transcriptome in the fish liver (a major metabolic site) and ovary (a reproductive organ, sensitive to estrogenic compounds). In the study, juvenile rainbow trout (*Oncorhynchus mykiss*) females were treated with ZEA at a dose of 10 mg/kg body mass for a short-term exposure period, i.e. 24, 72, and 168 h, respectively. DD-PCR was then used to generate a unique cDNA fingerprint of genes that were differentially expressed among the tissues of exposed and unexposed fish. Finally, Real-Time qPCR analysis was used to confirm the results and to obtain the expression profiles of genes indentified with DD-PCR. BLAST searches revealed the identity of isolated and confirmed transcripts, and the functional annotation provided additional information concerning the molecular background of ZEA action. To our knowledge, this is the first study documenting qualitative transcriptome changes in rainbow trout tissues after exposure to ZEA.

2. Material and methods

2.1. Fish exposure and samples collection

The fish were maintained in accordance with the regulations set out by the Local Ethical Commission No. 64/2008 issued on 18 September 2008 (conforming to the principles of Laboratory Animal Care; NIH, 1985). Juvenile all-female rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) individuals with an average body mass of 55.3 g and length of 17.9 cm were obtained from the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn, Poland). The fish were acclimatized for a minimum of 2 weeks prior to exposure. During the housing period, they were held in 800 L flowthrough tanks (wellwater, 600 L/h; photoperiod of 14/10) at 7 °C and fed four times a day to satiation. The trout were deprived of food 2 days prior to injections.

The set of treatment periods and doses of ZEA were based on our previous research (Woźny et al., 2008, 2010). Randomly sampled individuals were anesthetized by immersion in etomidate solution (Kazuń and Siwicki, 2001), and received single intraperitoneal injection of ZEA (at a dose of 10 mg/kg of body mass) dissolved in corn oil as a carrier solution, or corn oil alone (control sample). After 24, 72, or 168 h of exposure, 5 individuals were randomly taken from each experimental group, anesthetized, and decapitated by severance of the spinal cord. For the gene expression analysis, the ovaries and liver tips were excised and immediately immersed in RNA*Later*™ solution (Sigma; Germany) according to the manufacturer's recommendations, and stored at −20 °C.

2.2. Differential display PCR

To obtain the display of differentially expressed genes in rainbow trout tissues after exposure to ZEA, we used a method originally outlined by Sokolov and Prockop (1994). RNA*Later*™ preserved tissues (approx. 25 mg per sample; $n=3$ in each experimental group) were homogenized for mRNA extraction with PolyATract® System 1000 (Promega; USA) according to the manufacturer's protocol. The quality and quantity of isolated mRNA was estimated using a UV–Vis Biophotometer (Eppendorf; Germany). mRNA was used to synthesize cDNA using a commercially available RevertAid™ First Strand cDNA Synthesis Kit (Fermentas; Canada). The cDNA reaction for each sample contained 0.15 μg of high quality mRNA and 0.2 μg of random hexamer primers (RH); the reaction was performed according to the manufacturer's recommendations. Each synthesized first strand cDNA sample was incubated with 2 U of RNAse H (Promega) and then immediately used for PCR amplification (without freezing).

For the second step of the procedure, freshly synthesized cDNA samples were used as template for PCR amplification with defined but arbitrary decamer oligonucleotides (BS-52: 5′-CAA GCG AGG T-3′; BS-54: 5′-AAC GCG CAA C-3′; BS-55: 5′-GTG GAA GCG T-3′; BS-57: 5′-GAA AGC AGC T-3′; BS-58: 5′-CAG TGA GCG T-3′) paired in 10 primer combinations (52 + 54, 52 + 55, 52 + 57, 52 + 58, 54 + 55, 54 + 57, 54 + 58, 55 + 57, 55 + 58, 57 + 58). Each PCR reaction mixture contained 10 μL of 5× Go*Taq*® Flexi Green buffer (Promega), 1.5 mM of $MgCl_2$, 0.1 mM of dNTPs, 0.4 μM of each BS primer, 5 U of Go*Taq*® Hot Start Polymerase (Promega), 2 μL of cDNA template, and H_2O (PCR-grade) filled up to 50 μL of the final volume. The reaction was carried out on MasterCycler Gradient 5331 thermocycler (Eppendorf) using the following thermal profile: 95 °C for 2 min of initial denaturation, then 45 cycles of: 95 °C for 0.5 min, 34 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min of final elongation.

To generate a cDNA fingerprint, PCR products ($n=3$ in each experimental group) were electrophoresed in standard 1.5% agarose gel stained with ethidium bromide and visualized under UV light with a Gel Logic 200 Imaging System (Kodak; USA). Each differentially expressed cDNA band (Expressed Sequence Tag; EST) was isolated, purified with a Clean-Up kit (A&A Biotechnology; Poland), and reamplified using the same set of primers and original PCR conditions. Reamplified ESTs were once again purified (Clean-Up kit) and sequenced at the Institute of Biochemistry and Biophysics (Polish Academy of Sciences, IBB PAN; Poland). In the case of a low quality sequence electrophoregram and/or short reads (<100 bp), such an EST nucleotide sequence was removed from in silico analysis. BLAST queries were then performed to search for similarity of isolated ESTs to known sequences (Altschul et al., 1997) deposited in GenBank® databases (NCBI-NIH). BLAST matches were considered significant at the corresponding expect value of $E<10^{-5}$.

2.3. Real-time qPCR

In order to confirm the differentially expressed mRNAs, a real-time qPCR was used to examine the expression pattern of isolated ESTs in the same tissue samples but processed separately. Total RNA was extracted from the RNA*Later*™ preserved liver and ovary tissues (approx. 20 mg per sample; $n=5$ in each experimental group) using a Total RNA Mini isolation kit (A&A Biotechnology), according to the manufacturer's protocol. To prevent genomic DNA contamination, RNA samples were incubated with 5 U of RNase-free DNase I (Roche Diagnostics; Germany). Total RNA quality and quantity of all samples were estimated using a BioPhotometer (Eppendorf), and then used to synthesize cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The cDNA synthesis reaction mixture for each sample contained 1 μg of total RNA and 0.5 μg of oligo$(dT)_{18}$ primers; the reaction was performed according to the procedure recommended by the manufacturer. Synthesized first strand cDNA was suspended in sterile DEPC-H_2O and stored at −20 °C until used.

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