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Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (*Danio rerio*) exposed to water-soluble fraction of crude oil

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ABSTRACT

The induction of CYP enzyme activities, particularly CYP1A1, through the aryl hydrocarbon receptor (AhR) in most vertebrate species is among the most studied biochemical response to planar and aromatic organic contaminant exposure. Since P450 families play central roles in the oxidative metabolism of a wide range of exogenous and endogenous compounds, interactions between the biotransformation processes and reproductive physiological responses are inevitable. Steroidogenesis is the process by which specialized cells in specific tissues, such as the gonad, brain (neurosteroids) and kidney, synthesize steroid hormones. In the present study, we evaluated the effects of water-soluble fraction (WSF) of crude oil on the xenobiotic biotransformation and steroidogenic processes in the head (brain) and whole-body tissue of a model species by transcript analysis using quantitative (real-time) polymerase chain reaction (qPCR), enzyme activities and steroid hormone (testosterone: T and 17β-estradiol: E2) levels using enzyme immune assay (EIA). Our data showed that exposure of fish to WSF produced an apparent concentration-specific increase of AhR1, CYP1A1 and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA levels, and decrease of AhR2. On the activity level, WSF produced concentration-specific increase of ethoxyresorufin *O*-deethylase (EROD), benzyloxyresorufin (BROD) methoxyresorufin (MROD) and pentoxyresorufin (PROD) activities in whole-body tissue. In the steroidogenic pathway, WSF exposure produced apparent concentration-specific decrease of ERα and ERβ, steroidogenic acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450*scc*), P450*arom*A and P450*arom*B mRNA expression. For steroid hormones, while T levels decreased, E2 levels increased in an apparent WSF concentration-specific manner. In general, the xenobiotic biotransformation and estrogenic responses showed negative relationship after exposure of zebrafish to WSF, suggesting an interaction between these physiological pathways. The relationship between WSF mediated changes in brain StAR, P450*scc*, 3β-HSD, ERα, ERβ, P450*arom*A, P450*arom*B and whole-body steroid hormone levels suggests that the experimental animals might be experiencing altered neurosteroidogenesis probably through increased activity level of the biotransformation system. Thus, these responses might represent sensitive diagnostic tools for short-term and acute exposure of fish or other aquatic organisms to WSF.

1. Introduction

Environmental effects resulting from the offshore industry is an important topic in Norway. Oil exploration, production and transport pose potential risks to the marine environment (Horowitz et al., 1975; Sexstone and Atlas, 1977; Yarbrough and Chambers, 1977; Miller et al., 1978; Lavado et al., 2006; Lavarias et al., 2007). Norwegian authorities demand "zero-effect" discharges of oil in the North Sea and physical "zero discharges" in the Barernts Sea (white paper no. 58, The Norwegian Ministry of the Environment). To meet the requirements of "zero-effect" discharges, excellent and sensitive analytical tools are required for effect-determinations. In the past, effects from the gas and oil industry have been measured as lethality or by irreversible impairment by phenotypic methods (Depledge, 1984), but these are often not sensitive enough to measure effects from low exposure concentrations, and are difficult to use for long-term effects (e.g. generation-to-generation effects) (Moles, 1998). Current monitoring methods involve a combination of (a) chemical analyses of oil components and chemicals in sea water surrounding the platforms (Garcia de Oteyza and Grimalt, 2006); (b) measurements of oil components in wild fish and caged organisms around

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platforms (Booman and Føyn, 1996; Couillard et al., 2005); and (c) studies on stationary sea-bottom ecology around piles of cuttings below the platforms (Grigson et al., 2000). In addition to these field-based studies, laboratory studies using ecotoxicological tests are utilized to predict the toxicity of different compounds (Aas et al., 2000).

Xenobiotic biotransformation enzymes belonging to the P450 families play central roles in the oxidative metabolism of a wide range of exogenous and endogenous compounds (Nelson et al., 1996). For example, the CYP1, 2 and 3 enzyme superfamilies metabolize a wide variety of compounds, including oil-based compounds. The CYP1A have been detected in several organs and tissues, including brain, where its expression is regulated by the ligand-dependent basic helix–loop–helix–PER–ARNT–SIM (BHLH–PAS) transcription factor, aryl hydrocarbon receptors (AhR1 and AhR2) through which agonists cause altered gene expression and toxicity (Nelson et al., 1996; Bradshaw et al., 2002). Because of their roles in the detoxification and activation of foreign compounds, alteration of the expression of hepatic CYPs markedly affects the potential risks and benefits of xenobiotics and is important from a toxicological point of view (Williams et al., 1998).

Steroidogenesis is the process by which specialized cells in specific tissues, such as the gonad, brain and kidney, synthesize steroid hormones. Considerable diversity in specific steroids produced by different vertebrate groups or within classes of animals exists, but some generalization still applies (Stocco, 2000). For example, the steroidogenic acute regulatory (StAR) protein and P450*scc* (cytochrome P450 side-chain cleavage) are rapidly synthesized in response to acute tropic hormone stimulation and this is unique regardless of steroidogenic organ or tissue, The StAR protein transports cholesterol from the outer to inner mitochondrial membrane (Stocco, 2000). At the inner mitochondrial membrane, cholesterol is converted to pregnenolone by P450*scc* and pregnenolone is subsequently converted to progesterone by the 3[3-hydroxysteroid dehydrogenase (3β-HSD) enzyme (Kazeto et al., 2003). Neurosteroids are produced in the brain and function in stimulating and inhibiting gamma-aminobutyric acid receptor (GABAergic) responses, modulate the response of Purkinje cells to excitatory amino acids, and control memory (Flood et al., 1992). In vertebrates, the CYP19 is a crucial steroidogenic enzyme catalyzing the final step in the conversion of androgens to estrogens (Callard et al., 2001; Kishida and Callard, 2001). Teleost species have two structurally distinct CYP19 isoforms, namely, P450*arom*A and P450*arom*B. The P450*aromA* is predominantly expressed in the ovary and plays important roles in sex differentiation and oocyte growth, while P450*arom*B is expressed in neural tissues such as brain and retina and is involved in the development of the central nervous system and sex behaviors (Callard et al., 2001; Kishida and Callard, 2001).

At present, there is a general lack of knowledge on how marine organisms respond to oil discharges. Therefore, knowledge acquisition are urgently needed on (i) the molecular processes that can be linked to adverse effects in organisms exposed to acute and chronic oil compounds, (ii) how general expression patterns of proteins and mRNA change from a "normal state" during exposure and (iii) how molecular responses transcend to adverse effects at the population level. Modern molecular methods give possibilities for a better understanding of important processes and mechanisms involved in the generation of irreversible effects (from acute and chronic exposure) on individual-, species- and population-levels. These methods provide opportunities for identification of important biomolecules that can be used as biomarkers (either singly or in an entire biological system) for the prediction of oil discharge-related damage. Therefore, the aim of the present study is to evaluate the xenobiotic biotransformation and steroidogenic responses in the head region (brain) and whole-body tissue of zebrafish exposed to different concentrations of water-soluble fraction (WSF) of crude oil using molecular (mRNA), biochemical (enzyme activity) and physiological (hormone levels) methods. Our hypothesis is that fish exposure to WSF concentrations will produce differential and parallel response patterns of biotransformation and steroidogenic responses in the brain and whole-body tissues. These responses will represent sensitive and diagnostic biomarkers of effect and exposure to WSF and related compounds from the gas and oil industry.

2. Material and methods

2.1. Chemicals and reagents

Trizol reagent for RNA purification and TA cloning kit were purchased from Gibco-Invitrogen life technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from Sigma Chemical (St. Louis, MO, USA). Iscript cDNA synthesis kit and iTaq™Sybr® Green supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Generuler™ 100 bp DNA ladder and dNTPs are from Fermentas GmbH (Germany). E2 and T enzyme immuno-assay (EIA) kits (Cat. No. 582251 and 582701) were purchased from Cayman chemical company (Ann Arbor, MI, USA). All other chemicals were of the highest commercially available grade.

2.2. Generation of water-soluble fraction (WSF), fish and exposure

The oil used in the experiments was the *in reservoir* biodegraded Troll B crude oil. The oil was evaporated at 200 °C (200+ residue), which simulate a residence time in open water for 0.5–1 days (Daling et al., 1990). WSF was generated in a large glass flask (20 L) by careful application of oil on the surface of deionized water at an oil–water ratio of 1:100. The mixture was subjected to careful stirring (magnetic stirrer) at room temperature for 72 h. The WSF was then collected from a stop cock in the bottom of the flask and immediately used for exposure studies (Singer et al., 1998). The WSF contained approximately 2 mg/L oil compounds, as determined by gas chromatographic analysis and this was used as the 100% stock solution. Different (3%, 6%, 13%, 25%, 50% and 100%) dilutions were prepared from the 100% stock solution in individual reservoir flasks and were used in the flow-through experiments. A maximum number of six individuals of zebrafish were placed in each aquarium and exposed for 3 days (72 h). The flow-through rates were set to 1 L/min/kg fish in each aquarium to obtain enough oxygen saturation in the water. With six individuals per unit, this corresponded to a flow rate of 3 mL/min (each individual had an average weight of 0.5 g). Zebrafish (*Danio rerio*) is a useful model organism for studies of vertebrate development and gene function because they reproduce very easily, passing from egg to the larvae stage in less than 3 days. The experimental fish were supplied from a local pet shop with veterinary certificates and shored in 200-L aquaria with daily feeding of standard aquarium fish meal in a temperature controlled room at 28 °C.

2.3. Chemical analysis and Microtox bioassay

Total extractable organic carbon (TEOC) in WSF was determined by gas chromatography with flame ionization detector (GC-FID). The WSF was spiked with appropriate surrogate compounds and serially extracted with dichloromethane. The combined extracts were dried with sodium sulfate and concentrated to approximately 1 mL in a Turbovap. The final extracts were spiked with the appropriate recovery internal standards and analyzed by GC-FID for determination of total C_{10}–C_{36} (TEOC). GC-FID analysis were performed in a HP Model HP5890II gas chromatograph with a flame ionization detector (Agilent Technologies), using a Durabond DB-5 (15 m × 0.25 μm) column, hydrogen carrier gas (flow 2.2 mL/min), and a temperature program of 40 °C (5 min)–6 °C/min–310 °C (10 min) splitless in 40 s.

A closed-vial version of the Microtox® Acute Toxicity Test (AZUR Environmental Ltd, Carlsbad, CA) was performed as described in the manufacturers protocol.

2.4. Preparation of whole-body microsomes

Whole-body microsomal fractions were prepared by differential ultracentrifugation as described previously (Pesonen and Andersson, 1987). Whole-body samples (200 mg) from individual fish were sliced with a pair of scissors and homogenized in 1:4 ratio of body weight and volume of 0.1 M sodium phosphate buffer (containing