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Tissue-, sex- and development-specific transcription profiles of eight UDP-glucuronosyltransferase genes in zebrafish (*Danio rerio*) and their regulation by activator of aryl hydrocarbon receptor

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ABSTRACT

UDP-Glucuronosyltransferases (Ugts) are phase II biotransformation enzymes that glucuronidate numerous endogenous and xenobiotic substrates. Based on the reported zebrafish *Ugt* gene repertoire, primers for the *Ugt1a* and *Ugt1b* family and for individual *Ugt5a1*, *Ugt5a3*, *Ugt5a4*, *Ugt5a5*, *Ugt5c2* and *Ugt5c3* were designed and applied in RT-qPCR analyses. Transcriptional expression profiles of these *Ugts* were analyzed in intestine, liver, gonad and brain of female and male adult zebrafish and at different embryonic developmental stages. We found tissue-, sex- and developmental-specific expression patterns for all isoforms. Throughout all tissues, the most abundant *Ugts* were *Ugt1a*, *Ugt1b*, *Ugt5a1* and *Ugt5a3*. Expression during embryonic development was assessed between 24 and 120 hpf. *Ugts* showed a development-dependent expression. The pattern of *Ugt1a*, *Ugt1b*, *Ugt5a1*, *Ugt5a3* and *Ugt5a4* were similar with highest expression at 24 hpf followed by a decrease and rebound increase up to 120 hpf. To analyze for transcriptional regulation of *Ugts* by the arylhydrocarbon receptor (ahr2), zebrafish eleuthero-embryos were exposed to 5, 25 and 50 μg/L benzo(a)pyrene (BaP), a model ahr2 regulator for *cyp1a*. Besides transcriptional induction of *ahr2* and *cyp1a*, BaP produced a significant induction of *Ugt1a*, *Ugt5a1*, *Ugt5a3* and *Ugt5a5* as well as a down-regulation of *Ugt1b*. These data demonstrate the link between ahr2 signalling and transcriptional expression of *Ugt* genes. This is the first study showing transcriptional expression of eight different *Ugts* in tissues and during embryonic development and offers new perspectives on the involvement of *Ugts* in fish xenobiotic metabolism.

1. Introduction

UDP-glucuronosyltransferases (Ugts) are a supergene family of phase II drug-metabolizing enzymes, vital for detoxification of xenobiotics. They catalyze the conjugation of numerous hydrophobic small molecules with UDP-glucuronic acid converting them into hydrophilic molecules thus enhancing their water solubility and elimination. Ugts consist of many isoforms that can conjugate a broad range of both endogenous (bilirubin, bile acids, estrogens, androgens, thyroid hormones, etc.) and exogenous (phenols, non-steroidal anti-inflammatory drugs, etc.) substrates.

In humans and mammals *Ugts* are expressed in many different tissues, most dominantly in the liver, intestine and kidney. Exposure of animals with specific chemicals increases their expression and enzyme activity, hence they are induced similar to phase I enzymes including cytochrome P450 (Cyp) enzymes. The induction is based on the interaction with and stimulation of cellular receptors. Activation of different receptors by specific chemicals in mice including the aryl hydrocarbon receptor (Ahr), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator receptor α (PPARα), and the nuclear factor erythroid 2-related factor 2 (Nrf2) results in induction of distinct *Ugt* genes in liver and intestine (Buckley and Klaassen, 2009). Three or more *Ugt* genes were induced after activation of these receptors in the liver, most dominantly the *Ugt1a* family. Thus, activation of a single transcriptional pathway may lead to induction of many *Ugt* forms.

To date, very little is known about Ugts in fish. Recently the complete zebrafish *Ugt* gene repertoire was described (Huang and Wu, 2010). A total of 45 *Ugt* genes were identified that can be divided

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into three families consisting of *Ugt1*s, *Ugt2*s and *Ugt5*s. The *Ugt1* and *Ugt2* families have two unlinked gene clusters (a and b). Each of the *Ugt1a, Ugt1b, Ugt2a* and *Ugt2b* clusters contain variable and constant sequence regions. The *Ugt5* family exists only in lower vertebrates such as teleost fish and amphibians. This family is the most abundant *Ugt* family in fish. The evolutionary relationship of zebrafish *Ugt* genes has also been assessed, showing close similarities between mammalian and zebrafish *Ugt1* and *Ugt2* families (Huang and Wu, 2010).

The *Ugt* gene isoforms are distinct yet encode highly similar enzymes. The genes share large DNA sequence identities which are consequently seen as protein sequence identities, which makes identification and quantitation of specific gene transcripts by means of quantitative PCR (qPCR) challenging (due to the design of specific primers). Moreover, the transcriptional expression profiles of *Ugts* in tissues of zebrafish as well as in embryonic development stages are unknown. Additionally, very little is known on the regulation of specificity of *Ugt* forms by cellular receptors.

In aquatic organisms, *Ugts* are rarely assessed and discrimination of many different isoforms is lacking. Thus far, only one or a few isoenzymes of one *Ugt* family have been analyzed *in vivo* (Jones et al., 2010; Leaver et al., 2007; Mortensen and Arukwe, 2007a,b) or *in vitro* (Søfteland et al., 2010). In plaice and flounder, transcriptional tissue expression of *Ugt1b* was assessed (Leaver et al., 2007). Highest expression and induction of ugts was found in the liver and early embryonic stages. Plaice Ugt1b was found to be a phenol conjugating enzyme, but the substrate specificity or induction potential of different forms of *Ugts* are little known.

Herein we analyze transcripts of eight different *Ugts* in zebrafish with emphasis on the most abundant Ugt5 forms in fish by designing specific primers and detection by RT-qPCR. The aim of our work was to evaluate the transcriptional expression profile of different *Ugt* forms of all three *Ugt* families in different tissues of male and female zebrafish. An additional objective was to describe the transcriptional expression patterns of *Ugts* during early embryonic development. Transcriptional induction of *Ugts* via the aryl hydrocarbon receptor (Ahr) pathway was further analyzed in eleuthero-embryos after exposure to benzo(a) pyrene (BaP), a classical inducer of cytochrome P4501a (Cyp1a) activity (Bugiak and Weber, 2010; Otte et al., 2010). Zebrafish have three ahr (ahr1a, ahr1b, ahr2) forms (Evans et al., 2005), of which ahr2 is necessary for dioxin or polycyclic aromatic hydrocarbon toxicity (Prasch et al., 2003; Billiard et al., 2006). Zebrafish ahr2 is more closely related to the mammalian ahr than ahr1 (Andreasen et al., 2002). Presently, little is known on the role of ahr2 in the regulation of xenobiotic-inducible expression of different *Ugts*. Therefore we examined the transcriptional expression of *ahr1, ahr2* and *cyp1a*, the latter known to be up-regulated by the ahr2 pathway, to determine the associated *Ugt* forms.

2. Materials and methods

2.1. Chemicals

Acetonitrile, benzo(a)pyrene (BaP) (96% purity), bovine serum albumin, $CaCl_2x2H_2O$, dimethyl sulfoxide (DMSO), EDTA, EGF, 7-ethoxyresorufin (95% purity), HEPES, fluorescamine (98% purity), glucose, insulin, KCl, $MgSO_4x7H_2O$, NaCl, NADPH, $NaHCO_3$, $NaH_2PO_4{\cdot}xH_2O$, resorufin (95% purity) were purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2. Zebrafish cells and adult zebrafish

The *Danio rerio* ZFL cell line was obtained from the American Type Culture Collection (ATCC number CRL-2643). The medium consisted of 50% Leibovitz's L-15 medium (LuBioScience, Lucerne, Switzerland), 35% Dulbecco's modified Eagle's medium with 4.5 g/L glucose (LuBioScience, Lucerne, Switzerland), 15% Ham's F12 (LuBioScience) (all without sodium bicarbonate), and was supplemented with 0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL, insulin, 50 ng/mL EGF, and 5% heat-inactivated fetal bovine serum. ZFL cells were grown at 28 °C in a humidified incubator. Cells were usually split every 7 days and sub-cultured at split ratios of about 1:3.

Juvenile zebrafish were obtained from Harlan Laboratories (Itingen, Switzerland) and cultivated to adulthood in culture tanks (300 L). Fish of both sexes were held in reconstituted deionized water (salt concentrations: $CaCl_2x2H_2O$ 147.0 mg/L, KCl 2.9 mg/L, $MgSO_4x7H_2O$ 61.6 mg/L, $NaHCO_3$ 32.4 mg/L) with a conductivity of 350–360 μS/cm, and applying a static-water renewal procedure weekly. Water temperature was 26 ± 1 °C and the photoperiod 16:8 h light/dark. Fish were fed twice daily with a combination of frozen brine shrimps (*A. salina*), mosquito larvae and *D. magna*. Water parameters, such as nitrate, nitrite and pH were controlled regularly using Test strips (Easy Test, JBL) and oxygen was ensured to be ≥80% of air saturation.

2.3. Primer design

The NCBI tool for primer design was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast). For each primer pair, qPCR with a temperature gradient was run to identify the best annealing temperature. The obtained PCR products were loaded on an agarose gel to ensure that only one product was amplified. Additionally, the PCR products were sequenced by microsynth (http://www.microsynth.ch).

2.4. Sample preparation and SYBR Green qPCR

RNA from ZFL cells, five pooled zebrafish between 3 and 4 cm in size (intestine, liver, brain and gonads from females and males, respectively), and 15 pooled zebrafish eleuthero-embryos from different developmental stages (24 h, 48 h, 72 h, 96 h and 120 h) was isolated using RNA mini kit from Qiagen (Qiagen, Basel, Switzerland). RNA quality was assessed by Nanodrop analysis. In addition, agarose gels were run to test the integrity of the two RNA subunits. 1 μg of total RNA template was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C, 1 h at 37 °C, and the reaction was stopped by heating at 95 °C for 5 min. cDNA was used as a template to perform qPCR using SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland) and the primer pairs listed in Table 1. Real time PCR amplification was performed on a Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and annealing temperatures between 59 and 61.5 °C for 60 s, depending on the primer pair. Melting-curve analyses were performed after each run to ensure the formation of specific products. Quantification of *Ugt* transcripts were made using the relative quantification method and normalized to the housekeeping gene *RPL* with Qgene method (http://www.qgene.org/qgene/index.php) (Simon, 2003) according to the equation:

$$\mathrm{MNE} = \frac{E_{\mathrm{ref}} \wedge \mathrm{Ct}_{\mathrm{ref;mean}}}{E_{\mathrm{target}} \wedge \mathrm{Ct}_{\mathrm{target;mean}}}$$