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Biotransformation in the zebrafish embryo –temporal gene transcription changes of cytochrome P450 enzymes and internal exposure dynamics of the AhR binding xenobiotic benz[*a*]anthracene☆

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ABSTRACT

Not much is known about the biotransformation capability of zebrafish (*Danio rerio*) embryos. For understanding possible toxicity differences to adult fish, it might be crucial to understand the biotransformation of chemicals in zebrafish embryos i.e. as part of toxicokinetics.

The biotransformation capabilities were analysed for two different stages of zebrafish embryos in conjunction with the internal concentrations of a xenobiotic. Zebrafish embryos of the late cleavage/early blastula period (2–26 hpf) and the early pharyngula period (26–50 hpf) were exposed for 24 h to the AhR binding compound benz[*a*]anthracene (BaA). Time dependent changes in *cyp* transcription (*cyp1a, cyp1b1, cyp1c1 and cyp1c2*) as well as concentration & time-dependent courses of BaA in the fish embryo and the exposure medium were analysed. Additionally, the CYP mediated formation of biotransformation products was investigated.

We found correlations between transcriptional responses and the internal concentration for both exposure types. These correlations were depending on the start of the exposure i.e. the age of the exposed embryo. While no significant induction of the examined gene transcripts was observed in the first 12 h of exposure beginning in the blastula period a correlation was apparent when exposure started later i.e. in the pharyngula period. A significant induction of *cyp1a* was detected already after 1.5 h of BaA exposure. Gene transcripts for *cyp1b1*, *cyp1c1* and *cyp1c2* showed expressions distinctly different from *cyp1a* and were, in general, less inducible by BaA in both exposure windows. The toxicokinetic analysis showed that the biotransformation capability was fivefold higher in the older fish embryos. Biotransformation products of phase I reactions were found between 32 hpf and 50 hpf and were tentatively identified as benz[*a*]anthracene-phenol and benz[*a*]anthracene-dihydrodiol-epoxide.

In conclusion, not only duration but also onset of exposure in relation to the developmental stage of zebrafish embryos is important in the analysis and interpretation of effects due to different biotransformation capabilities.

1. Introduction

Aquatic organisms of different age and developmental stage are commonly exposed to low and sometimes transient concentrations of xenobiotics in the environment (Altenburger et al., 2015; Busch et al., 2016; Schwarzenbach et al., 2006). These xenobiotics can influence the homeostasis of adult organisms but also affect young -still developing-stages or organisms. The internal concentration of a xenobiotic is important as it reflects the fate of a xenobiotic in an

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organism and is a result of the sum of mass fluxes known as toxicokinetics (uptake, distribution, biotransformation and elimination) (Stadnicka et al., 2012). *In vitro* and *in vivo* studies in fish cell lines and fish respectively showed that bioconcentration/bioaccumulation and toxicity are in particular altered by xenobiotic biotransformation (Arnot et al., 2009; Kleinow et al., 1987; Nichols et al., 2007).

Biotransformation reactions are on the one hand described to protect the organism against adverse effects through detoxification and elimination of xenobiotics. A sufficiently high biotransformation & elimination rate can reduce the extent to which a xenobiotic accumulates in the exposed organism. The internal concentration i.e. the internal exposure therefore decreases over time and may result in a decline of toxicity. On the other hand biotransformation reactions may also alter the toxicity of xenobiotics by creating transformation products, which display either a lower or higher toxicity than the parent substance itself.

A well-known example for increasing toxicity through activation is the biotransformation of organophosphates or polycyclic aromatic hydrocarbons (PAHs). The transformation products of benz[*a*]pyrene, for instance, provoke carcinogenic, teratogenic and mutagenic effects (Buening et al., 1978; Gelboin, 1980; Shum et al., 1979). The exposure to PAHs induces the cytochrome P450 monooxygenase (CYP)[2] system through initial PAH binding to the aryl hydrocarbon receptor (AhR). The response compromises the induction of cytochrome gene expression and the follow up synthesis of cytochrome enzymes, which in turn catalyse the oxidative transformation of PAHs. Some of the resulting oxidized molecules have been shown to interact covalently with DNA, RNA and proteins and thus elucidating adverse effects.

The use of fish embryos has received increased attention since they are considered alternatives for experiments using adult fish and other vertebrates (Nagel, 2002). The zebrafish embryo in particular is an important model in embryology and has become popular in biomedical, ecotoxicological and toxicological research (Thienpont et al., 2011; Embry et al., 2010; Braunbeck et al., 2005; Langheinrich, 2003). The biotransformation capability (here meant purely qualitative i.e. the ability to biotransform xenobiotics) during the different pre adult stages of the zebrafish is dependent on the respective life stage (Knöbel et al., 2012; Weigt et al., 2011, 2010). The potential of zebrafish early life stages to perform biotransformation has been indirectly indicated at the gene transcription level (Goldstone et al., 2010) and directly on the enzyme activity via biotransformation product quantification (Brox et al., 2016; Yin et al., 2014; Busquet et al., 2008; Voelker et al., 2008) as well as on the toxicokinetic level (Kühnert et al., 2013). Biotransformation was also noted in exposed zebrafish embryos which were exposed to pro-teratogens which had to be biotransformed to have any effects (Weigt et al., 2011, 2010). With regard to the zebrafish Cyp enzyme system, which is capable to transform a large number of xenobiotics including PAHs and many other environmental pollutants, it is known that the expression of *cyp* genes varies during fish early life stages. In fact, many *cyp* genes show distinct temporal patterns of expression throughout early fish development (Goldstone et al., 2010). Hence, the biotransformation capability of fish early life stages can be assumed to be different from adult fish. Furthermore, the rate and extent of biotransformation might vary between the different fish early life stages and thus the respective toxic effects. To our knowledge, there is no study which links the time course of an induced *cyp* gene transcription to the exposure dynamics of AhR binding-xenobiotics in fish embryos.

We thus investigated the biotransformation capability of zebrafish embryos, explicitly addressing and comparing the temporal dynamics of biotransformation in two different early life stages of zebrafish. The magnitude and timing of xenobiotic induced transcription of different *cyp1* genes during early development of zebrafish are described and linked to the internal concentration over time. To this end, zebrafish (*Danio rerio*) embryos were exposed for 24 h to the known AhR agonist benz[*a*]anthracene (BaA), in a) an early exposure starting in the early blastula period (2–26 hpf) and b) in a later exposure starting in the pharyngula period (26–50 hpf). Moreover, Cyp mediated formation of biotransformation products was detected and temporal dynamics of these products in the late exposure experiments were investigated.

2. Materials and methods

2.1. Reagents

Benz[a]anthracene (BaA, CAS RN 56-55-3) was purchased from Aldrich (Lot MKBH3553V) with a purity of 99%. Acetonitrile (ACN, gradient grade) and HPLC water (ultragradient grade) were obtained from J.T. Baker (United States) and Merck (Germany), respectively. The test concentration of BaA was selected to be as high as possible in terms of water solubility. In a previous study performed under identical exposure conditions, no adverse acute effects were observed in embryos exposed to BaA up to the maximum of the water solubility (9.4 μg/L i.e. 41.2 nmol/L, according to EPIsuite, (US EPA, 2015; Kühnert et al., 2013). Thus, the stock solutions of BaA were freshly prepared for each experiment by adding an amount of BaA to standard dilution water (ISO water, as specified in the ISO 7346-3) which was slightly above known water solubility > 9.4 μg/L. This dispersion was stirred at room temperature for 24 h to solve as much as possible of BaA in the water within this time. After the stirring, the dispersion of the saturated solution was filtered through a Whatman® filter paper (Grade 114V) to prevent the transfer of non-dissolved solid BaA. The concentration was analytically measured immediately before using it as exposure solution. For the analyses of biotransformation products methanol (MeOH), Acetonitrile (ACN), ammonium acetate, and acetic acid (all UPLC/MS Grade) were purchased from Biosolve (Valkenswaard, Netherlands).

2.2. Danio rerio maintenance, embryo collection and exposure conditions

WIK zebrafish (*Danio rerio*) embryos were obtained from the Tübingen Zebrafish Stock Center (recently transferred to the European Zebrafish Resource Center, http://www.ezrc.kit.edu/). Zebrafish maintenance, embryo collection and staging were carried out as described in Kühnert et al. (2013) closely resembling standard procedures as specified in Westerfield (2000) and the OECD Technical Guideline 236 (OECD, 2013). During the 24 h exposure, the embryos were kept in a climatic chamber at 26 ± 1 °C with a 12 h light:12 h dark regime and were slowly agitated at 75 rpm (i.e. 0.1 *g*) using a horizontal shaker (Type SM-30-control, Edmund Bühler, Germany). The purpose of the slow agitation is the diminishment of the surrounding aqueous boundary layer of the chorion to accelerate transport of solutes through the chorion (Schreiber et al., 2009).

2.3. Experimental set-up

Zebrafish embryos were exposed before hatching to BaA for

[2] We use the designation of gene and protein names based on the Zebrafish Nomenclature https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines.