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Hypoxia and the pharmaceutical diclofenac influence the circadian responses of three-spined stickleback

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ABSTRACT

Pollution with low concentrations of pharmaceuticals, especially when combined with low-oxygen conditions (hypoxia), is a threat to aquatic ecosystems worldwide. The non-steroidal anti-inflammatory drug diclofenac is commonly detected in wastewater effluents, and has potential to accumulate in the bile of fish. Diclofenac has been shown to activate aryl hydrocarbon receptor (AHR), which induces transcription in the metabolic enzyme *cytochrome P450 1a* (*cyp1a*). Previously, crosstalk has been shown to occur between AHR and hypoxia inducible factor 1 (HIF-1). In addition, both of these transcription factors interact with the proteins regulating circadian (24-h) rhythms in vertebrates. Yet little is known about the significance of these interactions during simultaneous exposure to chemicals and hypoxia in fish *in vivo*. We exposed wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) to diclofenac (1 μg/L, 14 days), hypoxia (2.0 mg/L, up to 24 h) and the combination of both. We then analyzed markers of chemical biotransformation (EROD activity, *cyp1a* and *ahr* mRNA levels), glycolysis (lactate dehydrogenase (LDH) enzyme activity, *ldh* and *enolase 1a* mRNA levels), and the transcription of core circadian clock genes *clock* and *period 1* in liver tissue. Samples were taken at three time points during the light period in order to address disturbances in the circadian variation of metabolic processes. The results show that mRNA levels and LDH activity tended to be lowest before the dark period, but this pattern was disturbed by hypoxia and diclofenac. Diclofenac and hypoxia co-exposure induced EROD activity more strongly than diclofenac exposure alone, while *cyp1a* mRNA level was increased also by hypoxia and diclofenac alone. LDH activity and mRNA expression showed a clear time-dependent response during hypoxia, which is consistent with the previously suggested decreased accumulation of HIF-1 during the dark period. Furthermore, LDH activity and transcription was disturbed by diclofenac, indicating important effects of environmental pollutants in disturbing natural acclimation. This study demonstrates the need for more studies to understand the potential disturbances in endogenous rhythms caused by environmental pollution in natural populations.

1. Introduction

Increasingly, aquatic ecosystems are being stressed by pollutants, nutrient enrichment and global warming. A relatively new problem is micropollution, the chronic exposure to small concentrations of chemicals, including pharmaceuticals. This has been found to occur on several continents, and is dramatically on the rise (Loos et al., 2013; Luo et al., 2014; McEneff et al., 2014; Sim et al., 2013). What make pharmaceuticals problematic as pollutants are, firstly, their resistance to degradation whereby they pass wastewater treatment without being modified, and secondly, their targeted effects on specific biological pathways (Ankley et al., 2007). These pathways are often well conserved across phyla, and sensitive to exposure at extremely low doses (Ankley et al., 2007). Concurrently, rising temperatures and nutrient loads cause eutrophication, which depletes levels of dissolved oxygen, especially in freshwater systems and shallow coastal marine waters (Breitburg, 2002). Low-oxygen (hypoxic) conditions can have serious impacts on fish populations, contributing to habitat loss and periodic mass mortalities (Breitburg, 2002). Naturally, temperatures and oxygen levels oscillate in eutrophic environments: at

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night photosynthesis ceases, but organisms continue to consume oxygen, which causes hypoxia, while during the day, hyperoxia may occur together with higher temperatures. Despite the diurnal cycles of hypoxia, coupling of the hypoxia response to the circadian clock in fish has received little attention.

Among the commonly found pharmaceutical substances in wastewater effluents is diclofenac, a non-steroidal anti-inflammatory drug (NSAID) (Luo et al., 2014; Stulten et al., 2008). Diclofenac concentrations in wastewater treatment plants (WWTP) may vary, with effluents in Germany ranging from 1 to 2.2 μg/L (Luo et al., 2014), while those in a Greek river were on average 0.43 μg/L, with a maximum of 1.043 μg/L in the proximity of a WWTP (Stasinakis et al., 2012). In addition, Scheurell et al. (2009) described diclofenac concentrations in rivers in Pakistan ranging from 0.7 to 4.4 μg/L, exceeding a majority of the values reported from European WWTP effluents. Fluctuations in environmental concentrations of pharmaceutical effluents commonly occur due to variation in rainfall, wastewater treatment efficiency and human consumption (Tixier et al., 2003).

Diclofenac has been found to bioaccumulate in the bile of wild fish at 1000-fold concentrations compared to the environment (Brozinski et al., 2013). Similarly, bioaccumulation in the bile has been reported in laboratory conditions, with accumulation factors of roughly 500–600 (Mehinto et al., 2010). Brown et al. (2007) reported that bioaccumulation of diclofenac in rainbow trout blood plasma was low and shows significant spatial variation among sampling sites. Nevertheless, it is likely that excretion of diclofenac by fish is slow due to enterohepatic circulation. In previous studies, effects on gill, kidney and intestine morphology, as well as gene transcription in liver, were reported for exposure concentrations as low as 0.5–1.6 μg/L (Cuklev et al., 2011; Hong et al., 2007; Mehinto et al., 2010). Thus far, the combined effects of diclofenac and other environmental stressors on aquatic organisms have not been studied.

The key transcription factors in hypoxia responses (Hypoxia inducible factor 1, HIF-1), chemical biotransformation (Aryl hydrocarbon receptor, AHR), and the circadian clock (CLOCK and CYCLE–a.k.a ARNTL/BMAL1/MOP-3) are members of the Per-Arnt-Sim (Period-Arnt-Single minded, PAS) family of proteins (Hogenesch et al., 1998; McIntosh et al., 2010). The members of this family share a conserved basic helix-loop-helix (bHLH) and PAS amino acid sequences (Gu et al., 2000; McIntosh et al., 2010), which are important mediators of protein heterodimerization and DNA binding. Upon activation, the HIF-1 subunit HIF-1α and AHR dimerize with aryl hydrocarbon nuclear translocator (ARNT, also called HIF-1β). Thereafter HIF-1 and AHR exert their transcriptional effects by binding to their respective binding sites in the genome, thereby up-regulating the transcription of genes necessary for survival in hypoxic conditions, and for the biotransformation of several dioxin-like xenobiotics (Nikinmaa and Rees, 2005; Schmidt and Bradfield, 1996; Tijet et al., 2006).

During hypoxia energy is produced by glycolysis, since little oxygen is available for aerobic metabolism. In fish, several studies have shown that either the transcription or the activities of the glycolytic enzymes lactate dehydrogenase A (LDH) and enolase (ENO) are induced by hypoxia (Cooper et al., 2002; Dalla Via et al., 1994; Davies et al., 2011; Fonseca Almeida-Val et al., 2011; Roesner et al., 2006). Both enzymes have essential HIF-1 binding sites in their promoter regions in humans (Semenza et al., 1996). The target genes of AHR include metabolizing enzymes, such as Cytochrome P450 1A (CYP1A). CYP1A is a mixed function oxidase that plays an important role in the phase I biotransformation of xenobiotics (Andersson and Forlin, 1992). In fish, transcript levels of *cyp1a* have previously been shown to increase during exposure to diclofenac (Hong et al., 2007; Mehinto et al., 2010). In addition, several other pharmaceuticals have been shown to induce or inhibit CYP1A enzyme activity, measured as either ethoxyresorufin-*O*-deethylase (EROD) activity or gene transcription, suggesting CYP1A may have a significant role in their metabolism (Bartram et al., 2012; Beijer et al., 2013; Fernandez et al., 2013).

Circadian rhythms allow organisms to anticipate cyclical, daily changes in light, food availability, temperature and other *zeitgebers* (time-givers), and thus, allocate energy-demanding processes at the optimal occasions (Green et al., 2008). The transcriptional engine of the endogenous clock in vertebrates can be simplified into two feedback loops: a positive loop, involving the proteins CLOCK and CYCLE; and a negative loop, including the proteins CHRYPTOCHROME (CRY) and PERIOD (PER) (Lowrey and Takahashi, 2004; McIntosh et al., 2010). In the positive loop, the constitutively expressed CLOCK and CYCLE dimerize and bind to E-box elements in DNA, thereby regulating the expression of a variety of genes (Clock-controlled-genes; CCG), including *Per* and *Cry*. In the negative loop, PER and CRY proteins dimerize, and as they increase in abundance, inhibit the activation of the CLOCK-CYCLE-complex, and consequently their own expression, which ultimately allows the CLOCK-CYCLE dimer to continue DNA binding (Lowrey and Takahashi, 2004; McIntosh et al., 2010). The feedback loops oscillate in tissue-dependent rhythms in the central clock, which in fish is located in the pineal organ, and in the so-called peripheral clocks in other tissues (Idda et al., 2012; Vatine et al., 2011). The transcriptional clock is coupled to metabolism by sensing the reduced vs. oxidized state of cells with the help of a variety of enzymes (Edgar et al., 2012; Rey and Reddy, 2013).

Previously, a number of studies have addressed the interactions of hypoxia, AHR and circadian clock proteins. Hypoxia has been shown to affect transcription in the circadian clock genes *per1* and *clock* of zebrafish (Egg et al., 2013; Egg and Pelster, 2009). Egg et al. (2013) also showed that HIF-1 induction in hypoxia was dependent on the timing of hypoxia, and that hypoxia altered the rhythmic activity of the *per1* promoter in a zebrafish cell line. Further, Hogenesch et al. (1998) found that CYCLE (MOP-3) can act as a binding partner not only to CLOCK, but also to HIF-1α. Thus far, the interactions of AHR and circadian clock proteins have been described in mammals (e.g., Claudel et al., 2007), but not in fish. AHR activation was shown to disturb circadian clock gene transcription in murine hematopoietic progenitor cells (Garrett and Gasiewicz, 2006), and in mouse ovary and liver (Tischkau et al., 2011, Xu et al., 2010). In addition, the simultaneous activation of HIF-1 and AHR pathways have been shown to lead to preferential activation of the hypoxia response over chemical metabolism in fish (Fleming et al., 2009; Fleming and Di Giulio, 2011; Prasch et al., 2004).

To disentangle the functional significance of the interactions between concomitant activation of hypoxia and chemical responses and the circadian clock *in vivo*, we compared the enzyme and mRNA level responses between the beginning, middle and end of the light period using both single and combined exposures to hypoxia (2.0 mg O_2/L) and diclofenac (1 μg/L) in wild-caught three-spined sticklebacks (*Gasterosteus aculeatus* L.). Specifically, we investigated the following: (1) Is there diurnal variation in transcription and enzyme activities, and how do hypoxia and diclofenac treatments affect this variation? (2) Does the co-exposure to both hypoxia and diclofenac have effects that differ from single exposures? (3) How is the duration of hypoxia exposure reflected in the relationship of transcriptional and enzymatic responses?

2. Materials and methods

2.1. Conducting the experiment

Three-spined sticklebacks (*Gasterosteus aculeatus*, L.) used in the experiment were caught from the freshwater stream