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Clobetasol propionate causes immunosuppression in zebrafish (Danio rerio) at environmentally relevant concentrations

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ABSTRACT

Synthetic glucocorticoids (GCs) are potential endocrine disrupting compounds that have been detected in the aquatic environment around the world in the low ng/L (nanomolar) range. GCs are used as immunosuppressants in medicine. It is of high interest whether clobetasol propionate (CP), a highly potent GC, suppresses the inflammatory response in fish after exposure to environmentally relevant concentrations. Bacterial lipopolysaccharide (LPS) challenge was used to induce inflammation and thus mimic pathogen infection. Zebrafish embryos were exposed to ≤1000 nM CP from ~1 h post fertilization (hpf) to 96 hpf, and CP uptake, survival after LPS challenge, and expression of inflammation-related genes were examined. Our initial experiments were carried out using 0.001% DMSO as a solvent vehicle, but we observed that DMSO interfered with the LPS challenge assay, and thus masked the effects of CP. Therefore, DMSO was not used in the subsequent experiments. The internal CP concentration was quantifiable after exposure to ≥10 nM CP for 96 h. The bioconcentration factor (BCF) of CP was determined to be between 16 and 33 in zebrafish embryos. CP-exposed embryos showed a significantly higher survival rate in the LPS challenge assay after exposure to ≥0.1 nM in a dose dependent manner. This effect is an indication of immunosuppression. Furthermore, the regulation pattern of several genes related to LPS challenge in mammals supported our results, providing evidence that LPS-mediated inflammatory pathways are conserved from mammals to teleost fish. *Anxa1b*, a GC-action related anti-inflammatory gene, was significantly down-regulated after exposure to ≥0.05 nM CP. Our results show for the first time that synthetic GCs can suppress the innate immune system of fish at environmentally relevant concentrations. This may reduce the chances of fish to survive in the environment, as their defense against pathogens is weakened.

1. Introduction

Synthetic glucocorticoids (GCs) are commonly used in human and veterinary medicine for their immunosuppressive effects. GCs mimic the action of the steroid hormone cortisol, the natural stress hormone in humans and fish. They activate the glucocorticoid receptor (GR) and thus regulate the expression of GR-regulated genes playing a role in the immune system, but also development, glucose metabolism, osmoregulation, bone formation and behavior. Thus, GCs are potential endocrine disruptors: due to their hormone-like mode-of-action they may disturb the homeostasis of the stress-axis, i.e. the hypothalamus-pituitary-interrenal (HPI) axis in fish, and the hypothalamus-pituitary-adrenal (HPA) axis in mammals.

Kugathas and coworkers reviewed GC concentrations detected in the aquatic environment up to 2012, and used a mathematical model to predict the total environmental concentration of GCs in the River Thames (Kugathas et al., 2012). Based on their review and calculations, they proposed to conduct laboratory experiments assessing GC effects on aquatic organisms in the range between 10 and 1000 ng/L total GC. Indeed since then, several studies confirmed the presence of GCs around the world. The total detected GC concentrations were up to 57 ng/L in surface waters, up to 100 ng/L in wastewater treatment plant (WWTP) effluents and up to 836 ng/L in WWTP influents (Ammann et al., 2014; Herrero et al., 2014; Iglesias et al., 2014; Isobe et al., 2015; Jia et al., 2016; Liu et al., 2015; Macikova et al., 2014; Nakayama et al., 2016; Suzuki et al., 2015). Most of these studies used analytical procedures

Abbreviations: GC, synthetic glucocorticoid; GR, glucocorticoid receptor; CP, clobetasol propionate; WWTP, wastewater treatment plant; dpf, days post fertilization; hpf, hours post fertilization; BCF, bioconcentration factor; IS, internal standard; LPS, bacterial lipopolysaccharide; hpc, hours post challenge; DMSO, dimethyl sulfoxide; TLR, toll-like receptor

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that focus on individual compound detection.

GR-activation potential of single chemicals, chemical mixtures and environmental water samples can also be assessed using an *in vitro* assays, e.g. the GR-CALUX®, which measures the ability of all the compounds present in a sample to bind to and activate the GR (Van der Linden et al., 2008). Since this is a reporter gene assay, it indicates potential receptor-mediated effects, but may miss other impacts on the HPI axis. The potency of individual GCs to activate the GR varies, and it is expressed in relative potencies (REP) normalized to the GC dexamethasone. Application of this and similar assays detected GR-activity up to 2.7 ng/L dexamethasone equivalents (DEX EQs) in surface waters and up to 155 ng/L DEX EQs in WWTP effluents (Creusot et al., 2014; Jia et al., 2016; Macikova et al., 2014; Schriks et al., 2013; Stavreva et al., 2012; Suzuki et al., 2015; Van der Linden et al., 2008). Moreover, based on this approach, clobetasol propionate (CP) was shown to be one of the most potent GCs, having a REP of 38 (Macikova et al., 2014). CP itself was detected in many studies cited above at concentrations up to 1 ng/L in surface waters, 4.9 ng/L in wastewater treatment plant (WWTP) effluents, and 7 ng/L in untreated wastewater (Ammann et al., 2014; Isobe et al., 2015; Jia et al., 2016; Macikova et al., 2014; Nakayama et al., 2016; Suzuki et al., 2015). Hence GCs, such as CP, can reach the aquatic environment in significant concentrations and potentially adversely affect the aquatic biota. Fish and amphibians are most likely to be affected due to the conservation of major GR targets across vertebrates (Schaaf et al., 2009; Schoonheim et al., 2010; Steenbergen et al., 2011). Accordingly, Kugathas et al. showed that environmentally relevant concentrations of the GC beclomethasone dipropionate affected glucose metabolism and reproductive endpoints, and led to decreased blood lymphocyte count in fathead minnow (Kugathas et al., 2013).

However, the results obtained in most other studies examining GC effects in fish may have limited relevance for environmental risk assessment because much higher exposure concentrations than found in the aquatic environment were used (Gadan et al., 2012; Mathieu et al., 2013; Philip et al., 2012; Philip and Vijayan, 2015; Salas-Leiton et al., 2012; Sharif et al., 2015). Moreover, most reported studies used dimethyl sulfoxide (DMSO) as solvent carrier even though DMSO is known to have side-effects that may interfere with the endpoint of the assay used, and thus potentially affect the results (Oliveira et al., 2016; Santos et al., 2003). In this study, we attempted to address these aspects, using the zebrafish embryo, an excellent model for GC research (Alsop and Vijayan, 2009; Schaaf et al., 2009).

The innate immune system of zebrafish is active by one day post fertilization (dpf) (Herbomel et al., 1999), whereas the adaptive immune system is fully developed only 4–6 weeks post fertilization (Lam et al., 2004; Trede et al., 2004; Willett et al., 1999). Thus, using the zebrafish embryo model until 120 h post fertilization (hpf) for exposure experiments gives the opportunity to examine immune-related effects on the innate immune system alone. Moreover, using the zebrafish embryo model until 120 hpf, i.e. during the non-protected life stage, is considered an alternative to conventional animal testing (Strahle et al., 2012).

One of the important ways by which the innate immune system identifies pathogens is by recognizing pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Janeway, 1989). Bacterial lipopolysaccharide (LPS), a constituent of cell walls in Gram-negative bacteria is a common PAMP recognized by the PRR Toll-like receptor 4 (TLR4) in mammals (Poltorak et al., 1998). When LPS binds to TLR4, a pro-inflammatory cascade is activated, resulting in the release of mediators, like cytokines and chemokines. Myeloid differentiation primary response 88 (MyD88) is the adaptor protein of TLRs. In mammals, LPS-induced inflammation is regulated by MyD88-dependent and independent pathways. The dependent cascade regulates the early phase activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the mitogen-activated protein kinase (MAPK) pathways resulting in the release of inflammatory mediators, while the independent pathway involves the late phase activation of NFκB. Furthermore, it also activates the interferon (IFN) regulatory factor 3 (IFR-3) that leads to the production of IFN-β and thus expression of IFN-inducible genes (Takeda and Akira, 2004). It has already been shown that LPS induces the expression of key inflammatory genes in zebrafish embryos (Mottaz et al., in press; Novoa et al., 2009; Watzke et al., 2007). Indeed, the MyD88-dependent pathway is involved in the innate immune response of zebrafish embryos (van der Sar et al., 2006), and thus is a conserved pathway. Hence, challenging the embryos with LPS after exposure to a model GC and comparing the responses with the untreated control fish allows investigating whether GCs can cause immunosuppression in fish.

The aim of this study was to assess whether CP, an environmentally relevant GC that has a high potency to activate the GR, causes immunosuppressive effects in zebrafish embryos. The uptake of CP into embryos was also measured, and the bioconcentration factor (BCF) of CP for zebrafish embryos was determined. Gene expression analyses of genes involved in the anti-inflammatory action of GCs and/or the MyD88-dependent pathway were performed in order to investigate the molecular mechanism behind the observed inflammatory effects.

2. Material and methods

2.1. Chemicals

Clobetasol propionate (CP, CAS 25122-46-7), clobetasol (CAS 25122-41-2), hydrocortisone (CAS 50-23-7), lipopolysaccharide from *Pseudomonas aueruginosa*, NH_4HCO_3, dimethyl sulfoxide (DMSO), and the salts used to prepare the dilution water (294 mg/L $CaCl_2{\cdot}2H_2O$, 123.2 mg/L $MgSO_4{\cdot}7H_2O$, 64.74 mg/L $NaHCO_3$, and 5.75 mg/L KCl; prepared according to OECD test guideline 236) were purchased from Sigma-Aldrich (Buchs, Switzerland). The dilution water was prepared in MilliQ water. D9-progesterone (Progesterone-2,2,4,6,6,17a,21,21,21-d9, CAS 15775-74-3) was purchased from CDN Isotopes (Dr. Ehrenstorfer, Augsburg, Germany). Ethyl-acetate was from Merck (Zug, Switzerland), and HPLC-gradient pure acetonitrile (ACN) was from Acros Organics (Chemie Brunschwig AG, Basel, Switzerland).

2.2. Zebrafish husbandry and embryo collection

Adult fish from WiK (Max Planck Institute for Developmental Biology, Tübingen, Germany), OBI (Helmholtz Centre for Environmental Research established from OBI hardware store, Leipzig, Germany) and Qualipet (petshop, Switzerland) strains were maintained and crossed to have mixed genetic backgrounds and avoid possible inbreeding. The embryos resulting from these crossings were reared in our facility using commercial fish feed and were maintained according to recommended procedures (Nüsslein-Volhard and Dahm, 2002). Adult zebrafish were kept in a Mass Embryo Production System (Aquatic Habitats®, Pentair Aquatic Eco-Systems, Apopka, FL, USA) with a mix of tap and reversed-osmosis water (1:1) using a 14/10 h light/dark cycle at 28 °C. The embryo collection system was placed in the tank in the afternoon the day before collection. The embryos were collected 45 min after the light turned on in the morning, washed, and kept in dilution water (prepared according to OECD guideline 236). Next, the fertilized embryos were selected and placed in the pre-soaked wells or Petri dishes, depending on the experiment type.

2.3. Exposures

Approximately 0.5–1 mg CP was dissolved in one liter of dilution water and the concentration was measured by a UV–vis spectrophotometer (Cary 100, Agilent Technologies, Basel, Switzerland) at 240 nm wavelength. The stock solution was kept at 4 °C up to one week. Embryos were exposed to ≤1000 nM CP (≤46.7 μg/L) from ~1 hpf to 4 dpf at 28 °C using a 14/10 h light/dark cycle. For the uptake