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Oestrogen reporter transgenic medaka for non-invasive evaluation of aromatase activity

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ABSTRACT

Vertebrate reproduction involves complex steroid hormone interplay and inter-conversion. A critical element in maintaining sex steroid levels is the enzyme aromatase (cytochrome P450 19A1) which converts androgens to oestrogens. In turn oestrogen signalling is targeted by numerous chemicals, from pharmaceuticals to agricultural chemicals, both frequent sources of contamination in waste waters and consequently rivers. Although many models are now available to address disruption of oestrogen signalling, there are currently no published protocols allowing discrimination between alterations in testosterone metabolism and in oestrogenic signalling. It was with this limitation in mind that we optimised this protocol. We show using a 48 h protocol that pre-feeding fry of the *choriogenin h-gfp* (*chgh-gfp*) medaka line are sensitive to 0.05 nM EE2 (15 ng/L), within the range of the lowest published observable physiological effect concentrations for medaka. In addition, co-treatment with testosterone can reveal potential effects of test substances on aromatase enzymatic activity. As the measurements are visualised in real-time without affecting embryo viability, repeated measures are possible. We demonstrate the ability of this model to detect oestrogen receptor agonists, aromatisable androgens, P450 aromatase activity modulators and selective oestrogen response modulators. Importantly, the range of this assay is physiologically relevant.

1. Introduction

A major issue in environmental biology is modification of sex ratios in wildlife, as observed in animals ranging from invertebrates to amphibians, reptiles and mammals (Stoker et al., 2003; Brande-Lavridsen et al., 2008; Steinberg et al., 2008; Gagné et al., 2011). Such changes in sex ratios have been shown to be linked to the increasing presence of man-made chemicals in surface water (Larsson and Förlin, 2002; Hayes et al., 2010).

These findings underline the urgent need for physiological screening methods to identify endocrine disrupting chemicals acting on different levels of steroid signalling. To date the oestrogen axis has been the focus of most attention in this field due to its critical importance in cancer, reproduction, foetal development and sexual identity.

A diverse array of chemicals have been identified as affecting the oestrogen axis, ranging from phytochemicals to agricultural chemicals, pharmaceuticals, plasticisers, flame retardants, UV screens and industrial waste (Hokanson et al., 2007; Schmitt et al., 2008; Gyllenhammar et al., 2009; Kamata et al., 2011; Zhao and Mu, 2011; Edlow et al., 2012; Ezechiáš et al., 2012). In addition, population level effects of many of these substances have been proven, inducing effects as serious as intersex gonads in entire populations of male fish followed by a rapid decline in the population size (Jobling et al., 1998; Kidd et al., 2007).

However, in addition to identifying oestrogen axis disrupting chemicals active at the receptor level, it is also a priority to develop tests capable of detecting endocrine disrupting chemicals (EDCs) interfering with different aspects of steroid metabolism.

The enzyme aromatase (cytochrome P450 19A1) is responsible for the production of oestrogens from androgenic precursor molecules. It is therefore crucial in determining the relative levels of androgens and oestrogens in target tissues. It is now becoming clear that a number of environmentally relevant chemicals alter the activity of aromatase (Vinggaard et al., 2000; Hinfray et al., 2006). Recently developed

Abbreviations: Tam, Tamoxifen; Fad, Fadrozole; Dex, Dexamethasone; T3, 3,3′,5′-triiodo-L-thyronine; EE2, 17α-ethinyl oestradiol; E2, 17β-oestradiol; EC_{50}, Effective concentration 50 (half maximal effective concentration); T, Testosterone; SEM, Standard error of the mean; EDCs, Endocrine disrupting chemicals; ChgH, Choriogenin H; GFP, Green fluorescent protein; DMSO, Dimethyl sulfoxide; MS222, Ethyl 3-aminobenzoate methanesulfonate; dph, Day post hatch; ER, Oestrogen receptor; SERM, Selective oestrogen response modulator; LOEC, Lowest observed effect concentration; REACH, Registration, evaluation, authorisation & restriction of chemicals; OECD, Organisation for economic co-operation and development.

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in vitro tests for aromatase disruption, such as the human placental microsomal assay and the choriocarcinoma cell line assay, have proved to be useful screening tools for identifying aromatase disrupting chemicals (Snider and Brueggemeier, 1987; Brueggemeier and Katlic, 1990; Brueggemeier et al., 1997, 1992; Vinggaard et al., 2000). However, the lack of a simple and rapid *in vivo* test has made it difficult to determine the efficiency of these assays, which due to their *in vitro* nature fail to adequately take into account the complex nature of the reproductive axis, a system that involves interplay between numerous organs and crosstalk with other endocrine axes. In order to correctly evaluate exposure risks to humans and wildlife, it is important to distinguish between chemicals interacting with the oestrogen receptor and those altering the activity of aromatase.

The reaction of the European Union to the danger posed by EDCs has been to impose testing of all current and new chemicals produced in a quantity greater than one ton per year and suspected of being endocrine disruptors (REACH legislation). Considering the number of chemicals requiring testing, a screening test of at least medium throughput capacity is evidently required.

In this respect aquatic species such as medaka, zebrafish or premetamorphic *Xenopus* offer great promise. Their aquatic nature facilitates dosing, as the test substance can be added directly to the culture medium. Furthermore, the identification of the master sex determining gene in medaka (*dmy*) and more recently in *Xenopus laevis* (*dmw*) provides the additional advantage of allowing determination of the genetic sex of the test organism (Paul-Prasanth et al., 2006; Matsuda et al., 2007, 2002; Patil and Hinze, 2008; Yoshimoto et al., 2008). Coupled with the monitoring of gonadal status *via* biomarkers or histology, this allows cases of complete or partial sex reversal to be identified. At the time of writing, the master sex determining gene has recently been identified in zebrafish, although the study demonstrated that the gene is present on chromosome four in wild zebrafish, but is absent in laboratory strains (Wilson et al., 2014).

Many satisfactory oestrogenic biomarker genes have been identified (Trudeau et al., 2005; Kim et al., 2009; Chung et al., 2011; Gorelick and Halpern, 2011; Brion et al., 2012; O. Lee et al., 2012a, 2012b; W. Lee et al., 2012). In order to reveal the net effect over the entire oestrogenic pathway, and provide physiologically relevant information, the biomarker should be an endogenous gene relating to a physiological endpoint. Of this subset of identified genes, many are related to egg production, including *choriogenin h* (*chgh*) which codes for a structural protein of the egg envelope, produced in the liver (Ueno et al., 2004; Beck et al., 2005; Scholz et al., 2005; Zeng et al., 2005; Kurauchi et al., 2008, 2005; Salam et al., 2008; Chen et al., 2010). The OECD has acknowledged this viewpoint in a scientific review stating that the oestrogen sensitive protein *chgh-gfp* medaka embryo overcomes traditional limitations with the use of laborious whole organism tests, such as sensitivity and rapidity, as well as eliminating many ethical concerns (OECD, 2010).

Therefore, in order to fall in line with the three R's principal of reduction, replacement and refinement of animal experiments, these huge screening projects necessitate the use of non-regulated, early stage, pre-feeding fish fry or tadpoles. In particular, tests need to be ratified for their capacity to respond to a wide range of oestrogenic substances within a reduced time frame, ideally 24-48 h, and provide a sufficiently robust signal for robotised readout.

Here, we present an optimised, rapid 48 h protocol using pre-feeding *chgh-gfp* medaka for monitoring net oestrogen axis activity as well as the activity of the key enzyme P450 aromatase which controls the conversion of testosterone into 17β-oestradiol (E2). The activity of the oestrogen axis in the presence of test compounds was determined by the quantification of green fluorescent protein (GFP). Due to the low endogenous levels of testosterone in pre-feeding medaka fry, the readout of aromatase activity was achieved by co-treating fry with a known concentration of testosterone. Increases or decreases in the rate of conversion of testosterone to E2 resulted in a respective increase or decrease in the production of GFP under the control of the *chgh* promoter (Fig. 1).

Finally we present results generated with a number of reference compounds, selected in order to cover a range of mechanisms of action. We show that pre-feeding *chgh-gfp* medaka fry are a sensitive indicator of disruption of the steroid axes *via* a variety of mechanisms.

2. Materials and methods

2.1. Reagents

Ethyl 3-aminobenzoate methanesulfonate (MS222, CAS no. 886-86-2), dimethyl sulfoxide (DMSO, CAS no. 67-68-5), testosterone (CAS no. 58-22-0), 17α-ethinyl oestradiol (EE2, CAS no. 57-63-6), anastrozole (CAS no. 120511-73-1), 17β-oestradiol (E2, CAS no. 50-28-2), cromolyn (CAS no. 15826-37-6), saccharin (CAS no. 82385-42-0), cefuroxime (CAS no. 56238-63-2), arabinose (CAS no. 5328-37-0), amantadine (CAS no. 665-66-7), atenolol (CAS no. 29122-68-7), fadrozole (CAS no. 102676-31-3), aldosterone (CAS no. 52-39-1), progesterone (CAS no. 57-83-0), 3,3′,5′-triiodo-L-thyronine (T3, CAS no. 55-06-1), dexamethasone (CAS no. 50-02-2) and tamoxifen (CAS no. 10540-29-1). Medaka medium contained the following quantities of salts per litre: 10 g NaCl, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgSO4 \cdot 7H_2O$, 0.3 g KCl, 0.02 g methylene blue and its pH was adjusted to 7.2–8.0. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Transgenic line

All experiments were carried out with fry from generation F6 or greater of the WatchFrog *chgh-gfp* line. This line harbours a genetic construct consisting of 2047 bp of the medaka *chgh* gene immediately upstream from the translation start site driving expression of *gfp*, as initially described by Kurauchi et al. (2005). The line has a CarBio genetic background and displays a non-inducible basal fluorescence in cardiac muscle fibres and in certain cells around the mouth and eyes, allowing transgenic fry to be selected prior to exposure to potential EDCs.

2.3. Exposure studies

Stocks of test solutions were made up in DMSO and 8 μL of stock solution was added directly to 8 mL of medaka medium in 6-well plates. In addition either 8 μL of DMSO or 8 μL of testosterone in DMSO was added to each well. Final DMSO concentration was 0.2% in all wells. Five newly hatched (day post hatch zero; dph 0) medaka fry were added to each well and four wells were used for each exposure group giving a total of $n = 20$ fry per exposure group. All compounds were tested in the presence and absence of testosterone (28.57 μg/L). Three replicate experiments were carried out giving similar results, a representative replicate is shown. Solutions were renewed every 24 h and the experiments were read at 48 h (dph 2) with the exception of the experiment shown in Fig. 3. This experiment, to demonstrate the presence or absence of aromatase activity immediately after hatch was read after 24 h (dph 1).

2.4. Image capture

Chgh-gfp fry were anaesthetised with 200 mg/L MS222 in medaka medium and positioned dorsally on a black plastic plate for imaging. Images of the ventral region of the abdomen of each fry were captured with a 0.3 s exposure time at 8× magnification using an Infinity 1-3C camera (Lumenera Corporation, Ottawa, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and were stored as 8-bit colour jpg files. Larvae were illuminated with a 120 W fluorescence source and ET-GFP long-pass