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Transcriptional regulation of genes involved in retinoic acid metabolism in Senegalese sole larvae

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ABSTRACT

The aim of this study was the characterization of transcriptional regulatory pathways mediated by retinoic acid (RA) in Senegalese sole larvae. For this purpose, pre-metamorphic larvae were treated with a low concentration of DEAB, an inhibitor of RALDH enzyme, until the end of metamorphosis. No differences in growth, eye migration or survival were observed. Nevertheless, gene expression analysis revealed a total of 20 transcripts differentially expressed during larval development and only six related with DEAB treatments directly involved in RA metabolism and actions (*rdh10a*, *aldh1a2*, *crbp1*, *igf2r*, *rarg* and *cyp26a1*) to adapt to a low-RA environment. In a second experiment, post-metamorphic larvae were exposed to the all-*trans* RA (atRA) observing an opposite regulation for those genes involved in RA synthesis and degradation (*rdh10a*, *aldh1a2*, *crbp1* and *cyp26a1*) as well as other related with thyroid- (*dio2*) and IGF-axes (*igfbp1*, *igf2r* and *igfbp5*) to balance RA levels. In a third experiment, DEAB-pretreated post-metamorphic larvae were exposed to atRA and TTNPB (a specific RAR agonist). Both drugs down-regulated *rdh10a* and *aldh1a2* and up-regulated *cyp26a1* expression demonstrating their important role in RA homeostasis. Moreover, five retinoic receptors that mediate RA actions, the thyroid receptor *thrb*, and five IGF binding proteins changed differentially their expression. Overall, this study demonstrates that exogenous RA modulates the expression of some genes involved in the RA synthesis, degradation and cellular transport through RAR-mediated regulatory pathways establishing a negative feedback regulatory mechanism necessary to balance endogenous RA levels and gradients.

1. Introduction

Vitamin A (or retinol) and its derivatives (retinoids) are essential micronutrients that regulate a wide range of biological functions such as embryogenesis, vision, immune system, reproduction and skeletogenesis (Blomhoff and Blomhoff, 2006; Duester, 2008; Mora et al., 2008; Kam et al., 2012). This vitamin cannot be *de novo* synthetized in animals and thus needs to be obtained from the diet either as all-*trans*-retinol, retinyl esters or as provitamin carotenoid. Due to its important role in regulating several biological processes, vitamin A, unlike other vitamins, is stored as retinyl esters at relatively high levels mainly in the liver and secondarily in other extrahepatic tissues (O'Byrne and Blaner, 2013). The main bioactive metabolites derived from vitamin A are the 11-cis retinal and the retinoic acid (RA). The former mediates photoreception by acting as a visual chromophore while the later exerts a pivotal role controlling some regulatory pathways for cell differentiation, proliferation and apoptosis and thereby pleiotropic events in the embryonic development and adult physiology (Eroglu and Harrison, 2013; Doldo et al., 2015).

Vitamin A uptake and RA homeostasis are tightly regulated by evolutionary conserved mechanisms in vertebrates. The retinol is absorbed from the intestine into chylomicrons and stored mainly in the liver as retinyl esters. This hepatic retinol, when necessary, can be mobilized to the plasma and transported bound to the retinol-binding protein 4 (RBP4) to the target RA-generating cells. Once entered into the cell, the cellular retinol-binding protein (CRBP) facilitates its storage as retinyl esters or its conversion into all-*trans* RA (atRA) in a two-step oxidation reactions: i) from retinol to retinaldehyde, a reaction catalyzed by either alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs), and ii) from retinaldehyde to RA by retinaldehyde dehydrogenases (RALDHs). As RA acts primarily in a paracrine manner, the bioactive molecule is released to the surrounding environment where non-RA target cells degrade the molecule activating the cytochrome P450

Abbreviations: atRA, all-*trans* retinoic acid; CRBP, cellular retinol binding protein; CYP26, cytochrome P450; DEAB, 4-diethylaminobenzaldehyde; DMSO, dimethylsulfoxide; RA, retinoic acid; RAR, retinoic acid receptor; RBP, retinol binding protein; RDH, retinol deshydrogenase; RALDH, retinaldehyde dehydrogenase; RXR, retinoid X receptor; TTNPB, 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid; VA, vitamin A.

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enzymes (CYP26 family). On contrary, RA target cells express cellular RA-binding proteins (CRABP) that facilitate the transport to the nucleus to bind nuclear RA receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR), that, in turn, trigger many regulatory pathways with >500 target genes including hormones, growth factors, nuclear receptors, interleukin receptors, Hox genes and heat shock proteins (reviewed in Balmer and Blomhoff, 2002; Blomhoff and Blomhoff, 2006; Duester, 2008). RARs bind two active RA isomers, atRA and 9-*cis* RA, whereas RXRs appear to be activated exclusively by 9-*cis* RA (Duester, 2008).

Nutritional studies have demonstrated that fish larvae are highly susceptible to an unbalanced supply of vitamin A affecting growth and survival rates, performance (*i.e.* gut maturation) and malformation rates (Villeneuve et al., 2005; Fernandez et al., 2008; Mazurais et al., 2009). Particularly relevant is the impact of the hypervitaminosis A on larval morphogenesis that triggers the incidence of head, jaw, fin and vertebral malformations (Villeneuve et al., 2005; Villeneuve et al., 2006; Mazurais et al., 2009; Fernández and Gisbert, 2011). Some of these effects have been associated to abnormal atRA levels and a dysregulation of RAR-mediated signaling (Herrmann, 1995; Haga et al., 2002a; Haga et al., 2003; Villeneuve et al., 2005; Martinez et al., 2007). Moreover, atRA treatments can also induce pigmentation anomalies in flounders revealing a spatiotemporal window in RA sensibility during larval development (Haga et al., 2002b; Martinez et al., 2007).

The Senegalese sole (*Solea senegalensis* Kaup, 1858) is a high-value flatfish in fisheries and aquaculture. This species undergoes metamorphosis early during development for a short period of time (5–6 days), appearing as an excellent model to investigate regulatory pathways during development (Benzekri et al., 2014). However, larval quality, pigmentation and eye migration are affected by some environmental and nutritional factors (reviewed in Morais et al., 2016). Particularly, high dietary vitamin A levels trigger the incidence of skeletal deformities in post-larval specimens without detrimental effects on growth performance, metamorphosis progress, pigmentation pattern or survival. Interestingly, these larvae exhibited an increased production of thyroid hormones (THs), an essential molecule for sole metamorphosis (Manchado et al., 2008; Fernández et al., 2009). Also, dietary triacylglycerol levels modify the expression of genes involved in RA biosynthesis in pre-metamorphic larvae, probably modulating retinyl esters absorption (Hachero-Cruzado et al., 2014). In juveniles, high dietary vitamin A levels improve the immunocompetence and innate defenses against bacteria (Férnandez et al., 2015). However, a better knowledge of the mechanisms underlying RA production and signaling is required to understand the pleiotropic effects of retinoids and vitamin A in fish larvae. Thus, the aims of this work were: (i) to reveal the transcriptional regulation of genes involved RA biosynthesis, transport, and degradation in larvae exposed to atRA or an inhibitor of their synthesis; (ii) to determine the capability of atRA to regulate the expression of RAR and RXR receptors; and (iii) to identify the interaction between atRA and other hormonal pathways such as growth hormone- insulin-growth factor (GH-IGF) and hypothalamus-pituitary-thyroid (HPT) axes. To achieve these objectives, three experiments were carried out: 1) exposure of larvae to DEAB, an inhibitor of RALDH enzymes; 2) exposure of larvae to atRA; and 3) exposure to TTNPB, a specific RAR agonist to identify RAR-mediated effects. The results of this study are useful to understand the regulation of RA during larval development in sole.

2. Material and methods

2.1. Larval rearing conditions and fish samples

The study has been carried out in accordance with EC Directive 86/609/EEC for animal experiments. All procedures were approved with by IFAPA rules for the use of animals in research. Senegalese sole larvae were obtained from IFAPA center "El Toruño" (El Puerto de Santa Maria, Cádiz, Spain). The experimental design used in this study is depicted in Fig. 1.

Larvae were exposed to an inhibitor of RALDH, 4-diethylaminobenzaldehyde (DEAB; Sigma-Aldrich, ref. 31,830), to all-trans RA (atRA, Sigma-Aldrich, Ref R2625) which binds to RAR and RXR and to 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB, Sigma-Aldrich, ref. T3757), a specific agonist of the RARs. Solvent used were dimethylsulfoxide (DMSO; Sigma-Aldrich, ref. 472,301) for DEAB and TTNPB and ethanol (EtOH) for atRA. In control conditions, the same amounts of DMSO and/or EtOH were used as in exposed groups.

To optimize the drug treatments, a preliminary trial was carried out to determine the median lethal concentration (LC50) of DEAB in larvae at three ages: 3, 7 and 13 days post-hatch (dph). Larvae were exposed to increasing DEAB concentrations (0, 1, 10, 50, 100 and 200 μM) for 24 h (Supp. file 1). Treatments were carried out in duplicate using 2 L beakers containing 100 larvae with gentle aeration and supplied Artemia (1 metanauplii mL^{-1}) previously enriched with *Isochrysis galbana* (T-ISO strain) at exponential phase. The mortality was determined at 24 h after adding the drug by counting dead larvae and taking into account the mortality observed in the control by using the Abbott's formula: $(d_s - d_c) / (1 - d_c)$, where d_s and d_c are dead larvae from the sample and the control groups, respectively. The LC_{50} was calculated by using Probit analysis (Statplus®, AnalystSoft Inc., USA).

To evaluate the effect of DEAB (an inhibitor of retinaldehyde dehydrogenase 2 (*aldh1a2*), and, hence, of RA synthesis) on larval growth, survival rate and gene expression, 6 dph larvae were transferred to eight cylindrical conical tubes (volume 15 L) at an initial density of 60–70 larvae L^{-1} (Fig. 1). After 24 h (at 7 dph), half of tanks were supplemented with 10 μM DEAB and remaining tanks were used as controls. After adding DEAB, the water was kept stagnant for 24 h. Then, water was exchanged (20% per day) with the subsequent addition of DEAB or DMSO to maintain constant their final concentrations in the water. The physico-chemical parameters were as follows: temperature 19.8 ± 1.6 °C, salinity 36.1 ± 0.7‰, dissolved oxygen 6.2 ± 1.1 ppm, with gentle aeration in each tank. Larvae were fed twice a day with live preys keeping constant total volume as well as DEAB and DMSO concentrations. From 6 to 9 dph, rotifers *Brachionus plicatilis* enriched with microalgae (T-ISO strain) were provided. From 7 dph, larvae were also fed with increasing concentrations of *Artemia* metanauplii until the end of the experiment as described in Cañavate and Fernández-Diaz (1999). Thirty larvae per tank were randomly sampled at 6, 10, 12, 15, 19 dph for dry weight (DW) determination. Moreover, pools of thirty larvae per tank ($n = 4$) were collected at 9, 11 and 14 dph (2, 4 and 7 days after treatment (dat), respectively) for gene expression analysis. Larvae were washed using DEPC water, frozen in liquid nitrogen, and stored at −80 °C until use.

In a second experiment (Fig. 1), larvae from the previous experiment were exposed to atRA in order to rescue the inhibitory effect of DEAB on RA synthesis. For this purpose, 19 dph post-metamorphic larvae from the first experiment were pooled according to each treatment (DMSO or DEAB) and distributed into eight cylindrical tanks of 15 L (initial density: 5 larvae L^{-1}). After 24 h, DMSO-pretreated tanks were added DMSO and EtOH (DMSO + EtOH) in the control group or atRA (1 μM; atRA group) in duplicate. In a similar way, DEAB-pretreated tanks were added DEAB (10 μM; DEAB group) or DEAB (10 μM) and atRA (1 μM; DEAB + atRA group). The concentration of 1 μM atRA was established according to Higuchi et al. (2003) that observed an induction of gene expression in a time- and dose-dependent manner. Larvae were reared until 28 dph in similar conditions as those reported in the first experiment. Thirty larvae per tank were randomly sampled at 19, 26 and 28 dph for DW determination and two pools of larvae of each tank ($n = 4$) were collected at 22 and 26 dph (2 and 6 dat) for gene expression analysis. Larvae were washed using DEPC water, frozen in liquid nitrogen, and stored at −80 °C until use.

In order to check if the effects observed using atRA were throughout the RAR signaling pathway, a third experiment (Fig. 1) was carried out