Fish & Shellfish Immunology 38 (2014) 166–174

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Thyroid signaling in immune organs and cells of the teleost fish rainbow trout (*Oncorhynchus mykiss*)

A. Quesada-García [a], A. Valdehita [a], C. Kropf [b], A. Casanova-Nakayama [b], H. Segner [b], J.M. Navas [a,*]

[a] Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain
[b] Centre for Fish and Wildlife Health, University of Bern, Switzerland

ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form
6 March 2014
Accepted 12 March 2014
Available online 20 March 2014

Keywords:
Rainbow trout
Thyroid receptors
Immune cell markers
Triiodothryronine
Propylthiouracil

ABSTRACT

Thyroid hormones are involved in modulating the immune system in mammals. In contrast, there is no information on the role played by these hormones in the immune system of teleost fish. Here we provide initial evidence for the presence of active thyroid signaling in immune organs and cells of teleosts. We demonstrate that immune organs (head kidney and spleen) and isolated leukocytes (from head kidney and peripheral blood) of the rainbow trout (*Oncorhynchus mykiss*) express both thyroid receptor α (THRA) and β (THRB). Absolute mRNA levels of THRA were significantly higher than those of THRB. THRA showed higher expression in immune organs and isolated immune cells compared to the reference organ, liver, while THRB showed the opposite. *In vivo* exposure of trout to triiodothyronine (T3) or the anti-thyroid agent propylthiouracil (PTU) altered THR expression in immune organs and cells. Effect of T3 and PTU over the relative expression of selected marker genes of immune cell subpopulations was also studied. Treatments changed the relative expression of markers of cytotoxic, helper and total T cells (*cd4*, *cd8a*, *trb*), B lymphocytes (*mIgM*) and macrophages (*csf1r*). These findings suggest that the immune system of rainbow trout is responsive to thyroid hormones.

1. Introduction

Thyroid hormones (THs) play critical roles in growth, metabolism, and development in all vertebrates, including fish (reviewed in Ref. [53]). TH production is regulated by the hypothalamus–pituitary–thyroid (HPT) axis through positive and negative feedback mechanisms. In mammals, thyrotropin-releasing hormone (TRH) produced in the hypothalamus, stimulates the pituitary to release thyroid-stimulating hormone (TSH). However, in teleost fish this fact is less well established, and instead, corticotrophin-releasing hormone (CRH) appears to play an important role as a TSH-releasing factor (reviewed in Ref. [18]). TSH in turn, controls the secretion of L-thyroxine (T4) by thyroid follicles. Once in circulation, T4 enters target cells, where it undergoes monodeiodination, thus being converted into the biologically active 3,3′,5-triiodo-L-thyronine (T3). Correct function of the HPT axis is essential for the proper development and reproduction of animals (reviewed in Refs. [4,7]). In fish and amphibians, THs are responsible for the changes that occur during metamorphosis. For instance, the transformation of flounder larvae into juveniles has been shown to be dependent upon THs [52,53] and exposure to TH induces early metamorphosis in zebrafish [10].

In vertebrates, the action of THs is multifaceted and goes beyond development and reproduction [45,50,68]. One target of TH action appears to be the immune system. In mammals, there is a bi-directional and complex relationship between the HPT axis and the immune system (reviewed in Refs. [19,33]). For instance, the cells of the immune system produce TSH [64] and the presence of T3 has been reported in white blood cells and mast cells [16,48]. At the same time, thyroid receptors are present in mammalian immune cells, and THs have been found to influence the distribution of lymphocyte subsets [44,47] and to modulate specific immune responses, including cell-mediated immunity [9], B lymphocyte differentiations [46], natural killer cell activity, and T lymphocyte proliferation [27,54]. In addition, immune functions such as phagocytosis, the generation of reactive oxygen species (ROS), and the synthesis and release of cytokines are affected by hypo- and hyper-thyroid conditions (reviewed in Ref. [19]).

In teleost fish, THs have been studied mainly with regard to their function in development, metamorphosis, growth, and

* Corresponding author.
E-mail address: jmnavas@inia.es (J.M. Navas).

http://dx.doi.org/10.1016/j.fsi.2014.03.016
1050-4648/

reproduction (i.e.: [3,14,15,37]). In contrast, there is scarce information about a possible immunomodulatory role of THs in fish [70], although a few studies have provided indirect evidence that THs influence piscine immune parameters. Slicher [63] reported significantly reduced numbers of circulating leukocytes in hypothyroid fish. Correspondingly, Ball and Hawkins [1] observed that the administration of thyroxin or TSH recovered the number of circulating lymphocytes in hypo-physectomized killifish. More recently, an immunostimulating role of T3 has been suggested, based on the increased survival of T3-fed rohu (*Labeo rohita*) to *Aeromonas hydrophila* [59]. The involvement of THs in thymus development is supported by a study performed by Lam et al. [35]; who found that T4 increased thymus size in developing zebrafish, while the anti-thyroid agent methimazole caused the opposite effect. Information on a possible immunomodulatory role of THs in teleost fish is relevant with respect to comparative endocrinology and immunology. But, in addition, such information is significant with regard to potential impacts of thyroid-disrupting chemicals present in the environment (reviewed in Refs. [8,32,34]) to which natural fish populations and aquaculture facilities may be exposed [55].

THs exert their actions upon target organs and cells through binding to their receptors (THRs), which are ligand-dependent transcription factors that interact with specific DNA regions to regulate the expression of a wide range of genes. To date, two main subtypes of THRs, THRA and THRB have been identified in vertebrates. In fish, they are the product of at least two (i.e.: rainbow trout; [29,39] or three (i.e.: Japanese flounder; [71,72] distinct genes which can, in turn, generate several isoforms by alternate splicing.

In mammals, THRA and THRB are expressed in the organs and cells of the immune system (e.g.: Refs. [5,26,42]), and they mediate a number of immune effects exerted by THs [6,49]. Here we sought to unravel whether THRs are also expressed in immune organs and cells of teleosts and to evidence the effects of TH status on immune function. In this regard, we examined the following: (i) THRA and THRB expression in immune organs and cells of rainbow trout (*Oncorhynchus mykiss*); (ii) the levels and ratios of the two receptor subtypes expressed in these tissues and how they compare with the liver as a reference organ; (iii) alterations in immune THR expression in response to treatment with THs or anti-thyroid agents; and (iv) the association between these changes and altered ratios of marker genes of immune cell subpopulations.

2. Materials and methods

2.1. Fish samplings and T3 and PTU exposure experiment

Juvenile rainbow trout (*O. mykiss*, 49 ± 2.7 g body weight) were selected from the stock population reared at the Centre for Fish and Wildlife Health, University of Bern, Switzerland.

The fish used for exposure experiment were distributed into six glass aquaria, each with a volume of 200 L (ten fish per aquarium). They were kept under flow-through conditions (0.5 L/min) in air-saturated tap water (total hardness ($CaCO_3$): 2.13 ppm; chlorine dioxide: <0.01 mg/l) and at a water temperature of 15 ± 1 °C under a natural light–dark photoperiod.

Two groups (each with two replicates of 10 fish) were fed daily (1% weight) with a commercial diet spiked either with 20 μg/g of T3, or with 5000 μg/g of the anti-thyroid agent 6-Propyl-2-thiouracil (PTU). T3 and PTU were dissolved in ethanol and sprayed over the pellets, which were then dried at 60 °C to allow solvent evaporation. The control group (two replicates, each with 10 fish) was fed pellets exposed to the same percentage of ethanol. Test concentrations and duration of the exposure were selected following information available in the literature (For T3: Finsson and Eales [22] working on *O. mykiss*; Takagi et al. [66]; working on *O. mykiss*. For PTU: Peter and Peter [51]; working on tilapia (*Oreochromis mossambicus*); Sullivan et al. [65]; working on chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*), chinook (*Oncorhynchus tschawytscha*), and Atlantic salmon (*Salmo salar*). In those studies, the selected doses led to changes in plasma hormones (T3/T4) as well as in deiodinase activity and gene expression.

At the start of the experiments (day 0) nine animals were sampled for determining THR basal expression. Samplings of 10 fish per group (i.e. five fish per replicate) were performed at days 7 and 15 after the start of exposure, and blood and tissues of each two animals were pooled. Fish were immediately sacrificed with 100 mg/l of tricaine methane sulphonate (MS-222, Argent Chemical Laboratories, Redmont CA, USA). Animal fork length and mass were recorded and liver and gonads weighed. The hepatosomatic index (HI) was calculated following [24]. Immune cells from the head kidney and blood were isolated (see below), and RNA was immediately extracted. Samples from the liver, head kidney, and spleen were stored in RNA-later (Sigma–Aldrich, Buchs, Switzerland) until RNA extraction.

2.2. Leukocyte isolation

Immune cells from the head kidney and blood were isolated using the Ficoll-Hypaque method (Biochrom AG, Berlin, Germany). Briefly, 1 mL of blood (pool of 500 μL from two animals belonging to the same group) withdrawn from the caudal vein was diluted 1:10 with L-15 medium (Gibco, Basel, Switzerland) containing 10 U/mL of heparin. In the case of the head kidney, approximately 0.5 mg of tissue (pool of two animals belonging to the same group) was homogenized by repeated pipetting with 10 mL of L-15, and the resulting homogenate was passed through 250-μm and 125-μm nylon meshes. Ten mL of the resulting cell suspension (either blood/L-15 or head kidney/L-15) was then placed onto 3 mL of Ficoll (1.077 g/ml; Biochrom AG, Berlin, Germany) and centrifuged for 40 min at 400 × *g* at 4 °C. The layer containing the leukocytes was transferred to a fresh tube and washed several times by centrifugation (10 min at 400 × *g* 4 °C). The final pellet was lysed in TRI® reagent and maintained at −80 °C until RNA extraction.

2.3. RNA extraction and synthesis of first strand cDNA

Total RNA was extracted from tissues and isolated immune cells using TRI® reagent, following the manufacturer's instructions (Sigma–Aldrich, Buchs, Switzerland). After DNA digestion with RQ1 RNase-Free DNase (Promega AG, Wallisellen, Switzerland), RNA concentration and purity was measured with NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington DE, USA). Subsequently, reverse transcription (RT) was carried out in 20 μL of reaction volume containing 500 ng Random Primers, 2.5 mM $MgCl_2$, 2 mM dNTPs, 20 U RNAse inhibitor, 160 U M-MLV reverse transcriptase (all Promega AG, Wallisellen, Switzerland) and 500 ng total RNA. cDNA was kept at −20 °C until quantitative PCR (qPCR) was performed.

2.4. PCR primers

Primers for THRs (*thra* and *thrb*) and deiodinase type 2 (*dio2*) were designed using sequences obtained from GeneBank (NCBI). As immune markers, T cell receptor, beta chain (*trb*), *cd4* and *cd8a* were selected to monitor changes in T cell dynamics [56]. T-cell receptor is expressed on the surface of all T cells, while CD8α and CD4 distinguish between subpopulations of T cells (cytotoxic T cells and helper T cells, respectively). Macrophage Colony-Stimulating