General and Comparative Endocrinology 225 (2016) 142–148

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Retinoid level dynamics during gonad recycling in the limpet *Patella vulgata*

M. Gesto [a,b,1], R. Ruivo [a,1,*], I. Páscoa [a], A. André [a,c], L.F.C. Castro [a,d], M.M. Santos [a,d,*]

[a] CIMAR/CIIMAR-Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal
[b] Laboratorio de Fisioloxía Animal, Facultade de Bioloxía, Universidade de Vigo, 36310 Vigo, Spain
[c] ICBAS – Institute of Biomedical Sciences of Abel Salazar, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[d] FCUP – Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

ARTICLE INFO

Article history:
Received 16 June 2015
Revised 14 October 2015
Accepted 22 October 2015
Available online 24 October 2015

Keywords:
Evolution
Gametogenesis
Invertebrates
Nuclear receptors
Retinoid pathways

ABSTRACT

Germ cell commitment and meiosis initiation are among the multitude of physiological roles of retinoic acid (RA) in vertebrates. Acting *via* receptor-mediated transcription, RA induces the expression of meiotic factors, triggering meiosis. Contrasting with vertebrates, invertebrate RA metabolism is scarcely understood. Still, some physiological processes appear to be conserved. Here we set to evaluate the role of retinoids in the gonad maturation process of the marine gastropod *Patella vulgata*. We found that retinoid concentration in gonadal tissue, namely RA, varies between breeding and resting specimens, with maxima attained in the latter. Additionally, we isolated and quantified the expression of both the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) in gonads. In view of the stability of retinoid receptor expression, we suggest that the balance of RA levels operates through the enzymatic control of synthetic and catabolic processes. Overall, the reported data are supportive for a developmental role of RA during gonadal maturation in *P. vulgata*, which should be addressed in other protostome lineages.

1. Introduction

Retinoids are vitamin A (retinol)-derived lipophilic molecules that include a biologically active metabolite, retinoic acid (RA). Vertebrate RA signaling contributes to an array of physiological processes in embryo and adult tissues: from development and growth to tissue maintenance, vision and reproduction (Andre et al., 2014; Blomhoff and Blomhoff, 2006; Clagett-Dame and DeLuca, 2002; Maden, 2007; Theodosiou et al., 2010). RA was also suggested to play a key role in sex-dependent germ-cell fate in mammals, birds, teleosts and anurans, acting as a molecular cue required for meiosis initiation (Bowles et al., 2009, 2006; Bowles and Koopman, 2007; Piprek et al., 2013; Rodriguez-Mari et al., 2013; Smith et al., 2008). Regardless of sex-determining mechanisms, meiotic progression differs between sexes. For instance, in mammals, egg-producing germ cells initiate meiosis during embryonic development whereas in males meiosis is halted until birth (Bowles et al., 2006). In the teleost *Danio rerio*, presenting no apparent heteromorphic sex chromosomes and a bipotential default ovary, a similar pattern was observed: upon sexual differentiation meiotic schedule is dimorphic (Rodriguez-Mari et al., 2013; Traut and Winking, 2001). To dictate meiotic arrest or progression, RA was suggested to regulate the expression of genes coding for several proteins of the meiotic machinery (Bowles et al., 2006). In fact, vertebrate RA signals through two nuclear receptors, mediating the transcription of target genes: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Theodosiou et al., 2010). RAR and RXR form a heterodimeric nuclear receptor complex, with RXR serving as obligate partner to RAR (Andre et al., 2014; Theodosiou et al., 2010). Two RA isomers, all-*trans*-RA and 9-*cis*-RA, serve as high affinity ligands for RARs whereas only 9-*cis*-RA binds with high affinity to RXRs (Blomhoff and Blomhoff, 2006).

Abbreviations: BLAST, basic local alignment search tool; CYP26, cytochrome P450 family 26; HNF4, hepatocyte nuclear factor 4 receptor; HPLC, high-performance liquid chromatography; HPLC–MS, high-performance liquid chromatography–mass spectrometry; LC/MS/MS, high-performance liquid chromatography–tandem mass spectrometry; RA, retinoic acid; RAR, retinoic acid receptor; RACE, rapid amplification of CDNA ends; RE, retinyl ester; ROL, retinol; RP, retinyl palmitate; RXR, retinoic X receptor.

* Corresponding authors at: CIMAR/CIIMAR, LA-Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal (M.M. Santos).

E-mail addresses: rruivo@ciimar.up.pt (R. Ruivo), santos@ciimar.up.pt (M.M. Santos).

[1] Equal contribution.

http://dx.doi.org/10.1016/j.ygcen.2015.10.017
0016-6480/

Previously believed to be a vertebrate innovation, several molecular components of retinoid signaling and metabolism have now been found in other bilaterian lineages (Albalat and Canestro, 2009; Andre et al., 2014; Theodosiou et al., 2010). Regarding retinoid active molecules, polar RA isomers have been identified in different tissues of several invertebrate species (Albalat, 2009; Andre et al., 2014; Theodosiou et al., 2010). Nonpolar retinoids, such as retinol (ROL) or retinyl esters (RE) have been reported mainly in the gonad (Andre et al., 2014; Theodosiou et al., 2010). In vertebrates, RE and ROL are typical storage and transport forms which undergo de-esterification and oxidation, respectively, serving as sequential precursors in the biosynthesis of RA (Andre et al., 2014; Theodosiou et al., 2010). Additionally, esterified retinol, mostly in the form of retinyl palmitate (RP), participates in the homeostatic regulation of retinoid levels, modulating their availability (Andre et al., 2014; Gesto et al., 2012a). Further RA oxidation leads to the inactivation of the signaling molecule (Theodosiou et al., 2010). Although invertebrate retinoid physiology is poorly understood, several lines of evidence put forward some evolutionary conserved processes (Albalat and Canestro, 2009; Dmetrichuk et al., 2008). For instance, similarly to vertebrate RA, retinoids drove neuronal outgrowth in the gastropod *Lymnaea stagnalis* (Dmetrichuk et al., 2008; Maden, 2007). In gastropods, several reports also suggest a role of RA signaling in the development of reproductive structures in different species: *Thais clavigera*, *Nucella lapillus* and *Ilyanassa obsoleta* (Castro et al., 2007; Nishikawa et al., 2004; Sternberg et al., 2008). Recent studies have also described a significant retinoid content in male gonads of *Osilinus lineatus* and *Patella depressa*, suggestive of a role in testis development and/or reproductive processes (Gesto et al., 2012a, 2013). This hypothesis is further corroborated by the observed positive correlation between nonpolar retinoid concentration in the digestive gland/gonad complex and male gonadal maturation stage in *O. lineatus* (Gesto et al., 2012a). However, for a yet unknown reason, storage retinoids are apparently absent in some gastropod species (Gesto et al., 2013).

The genetic machinery related to retinoid function has also been documented outside vertebrates. Genes coding for RXR are generally present in bilaterians, while RAR genes though present in lophotrochozoans (e.g. annelids and molluscs) are absent in ecdysozoans (e.g. insects and nematodes) (Albalat and Canestro, 2009; Andre et al., 2014; Theodosiou et al., 2010). Regarding molluscs, functional RXR orthologs were reported in various species such as *T. clavigera*, *Biomphalaria glabrata*, *N. lapillus, L. stagnalis* and *I. obsoleta* (Bouton et al., 2005; Carter et al., 2010; Castro et al., 2007; Nishikawa et al., 2004; Sternberg et al., 2008); to date, RAR was only isolated from *N. lapillus*, *T. clavigera* and *L. stagnalis* (Carter et al., 2015; Gutierrez-Mazariegos et al., 2014; Urushitani et al., 2013). However, while RXR ligands appear highly conserved across phyla, *N. lapillus* and *T. clavigera* RAR were shown to be unresponsive to retinoids (Gutierrez-Mazariegos et al., 2014; Urushitani et al., 2013).

Given the prominent role of retinoids in gametogenesis across vertebrate phyla, we aimed at addressing the involvement of the retinoid system, that is retinoid metabolism and signaling, during gonad maturation in an invertebrate group, the gastropods. For the present study, we selected the limpet *Patella vulgata* (Linn. 1758) based on two reasons. First, this species was expected to have a complex retinoid system, with both polar and nonpolar retinoids, since both were previously detected in a related species, *P. depressa* (Gesto et al., 2013). Secondly, unlike other limpets, *P. vulgata* has a highly synchronized reproductive cycle, with well-defined breeding and resting periods, facilitating the collection of homogeneous specimens, in terms of size and reproductive stage (Ribeiro et al., 2009). After gamete discharge, gonads from both males and females regress to an immature-like or resting spent stage (neuter animals). During the inter-spawning intervals a novel cycle of gamete maturation is resumed (Orton et al., 1956). Thus, we have assessed the gonadal retinoid content in breeding and resting specimens of *P. vulgata*. Additionally, the genes coding for *P. vulgata* RAR and RXR retinoid receptors were cloned and their expression quantified in gonads at different reproductive stages.

2. Materials and methods

2.1. Animal sampling

A total of 63 limpets (mean shell length: 29.7 ± 3.27 mm; mean body mass without shell: 1714.3 ± 673 mg) were sampled in Apúlia, Portugal, in July 2010. All specimens were in the resting condition (neuter gonad, stage 0 according to Orton et al. (1956)), in accordance to the breeding cycle of Portuguese *P. vulgata* described by Ribeiro et al. (2009). In October 2010, 18 more limpets (mean shell length: 31.1 ± 2.9 mm; mean body mass without shell: 2074.8 ± 806.1 mg), 10 mature females (full gonad, stage 4–5) and 8 mature males (full gonad, stages 4–5, Orton et al., 1956), were collected at the same place. After capture, animals were transported to the laboratory and the gonad was immediately sampled and stored at −80 °C. Due to the small size of the neuter gonads (6.5 ± 2.5 mg), they were pooled together (4 pools of 10–17 gonads). All experiments complied with European Guidelines for the correct use of laboratory animals. Before sacrifice and tissue sampling, animals were always sedated in a 7% magnesium chloride solution for 5 min.

2.2. Analysis of retinoid content

Extraction of retinoids from tissues was carried out as previously described (Gesto et al., 2012a). HPLC analysis of free ROL, total ROL (free + esterified) and retinyl palmitate (RP) was carried out according to (Gesto et al., 2012b). For RA quantification, three pools of each group were submitted to AS Vitas (Oslo, Norway), to be analyzed by LC/MS/MS (Gundersen et al., 2007): two gonad pools for mature females and males and pools of 10–28 gonads for neuter animals. Statistical analysis was performed using the software SigmaPlot 11.0. After testing ANOVA assumptions, RA levels were analyzed through a one-way ANOVA followed by the Tukey post hoc test.

2.3. Tissue RNA extraction

Total RNA extraction from limpet gonads was performed with a combination of methods. First, tissues were homogenized with TRIzol reagent® (Invitrogen) and nucleic acids extracted with chloroform, according to the manufacturer's instructions. The resulting aqueous phase was used to isolate total RNA using the Illustra RNAspin Mini RNA Isolation Kit animal tissues protocol (GE Healthcare) with on-column DNase I digestion. RNA quality was assessed by electrophoresis and its concentration determined by fluorescence (Fluoroskan Ascent, Labsystems) using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen). First-strand cDNA was synthesized from 350 ng of total RNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions.

2.4. pvRAR and pvRXR isolation

P. vulgata RAR and RXR orthologues were isolated using a combination of PCR-based approaches. First, degenerate PCR primers were designed from conserved regions of both nuclear receptors (Supplementary Table 1). To extend the initial sequence RACE-ready cDNA was synthesized using the SMARTer™ RACE cDNA