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Short Communication

Thyroid hormone deiodinase type 2 mRNA levels in sea lamprey (*Petromyzon marinus*) are regulated during metamorphosis and in response to a thyroid challenge

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ABSTRACT

Thyroid hormones (THs) are crucial for normal vertebrate development and are the one obligate morphogen that drives amphibian metamorphosis. However, contrary to other metamorphosing vertebrates, lampreys exhibit a sharp drop in serum TH early in metamorphosis, and anti-thyroid agents such as potassium perchlorate ($KClO_4$) induce metamorphosis. The type 2 deiodinase (D2) enzyme is a key regulator of TH availability during amphibian metamorphosis. We set out to determine how D2 may be involved in the regulation of lamprey metamorphosis and thyroid homeostasis. We cloned a 1.8 Kb *Petromyzon marinus* D2 cDNA that includes the entire protein coding region and a selenocysteine (Sec) codon. Northern blotting indicated that the lamprey D2 mRNA is the longest reported to date (>9 Kb). Using real-time PCR, we showed that intestinal and hepatic D2 mRNA levels were elevated prior to and during the early stages of metamorphosis and then declined dramatically to low levels that were sustained for the remainder of metamorphosis. These data are consistent with previously reported changes in serum TH levels and deiodinase activity. Treatment of larvae with either TH or $KClO_4$ significantly affected D2 mRNA levels in the intestine and liver. These D2 mRNA levels during metamorphosis and in response to thyroid challenges suggest that D2 may function in the regulation of TH levels during lamprey metamorphosis and the maintenance of TH homeostasis.

1. Introduction

Thyroid hormones (THs) regulate a variety of different biological systems in invertebrates and vertebrates, notably their development [17,27,34]. In amphibians and fish, peak serum TH concentrations coincide with the period of greatest metamorphic change [27,34]. THs are also pivotal in lamprey metamorphosis, although their involvement appears somewhat paradoxical to their role in other metamorphosing vertebrates (reviewed in [27]). The lamprey lifecycle begins with a fresh-water, filter-feeding larval phase followed by a true metamorphosis, which gives rise to a free swimming parasitic juvenile. Lamprey metamorphosis involves numerous, dramatic internal and external changes including, degeneration (e.g. larval oesogphagus and bile ducts) or remodelling (e.g. oral apparatus, branchiopores) of existing structures and *de novo* synthesis of organs from undifferentiated tissue (e.g. opisthonephric kidney, eyes; reviewed in [46]). In sea lampreys (*Petromyzon marinus*), serum TH levels gradually increase over the course of the 2–7 year larval feeding period, peak prior to metamorphosis, then drop sharply by the 2nd or 3rd stage of metamorphosis [23,41,44]. Treatment of larval lamprey with the iodide uptake (TH synthesis) inhibitor potassium perchlorate ($KClO_4$) lowers TH levels and induces precocious metamorphosis. $KClO_4$-induced metamorphosis is completely blocked by exogenous TH treatment [18,24,25]. The lamprey response to TH inhibitors and to exogenous treatment with THs is opposite to that of other metamorphosing vertebrates, thus the role of THs in regulating lamprey metamorphosis has long puzzled researchers.

The synthesis of THs by thyroid tissues is under the control of the hypothalamic-pituitary axis, with T_4 (thyroxine, 3′,5′,3,5-L-tetraiodothyronine) being the primary secretory product. The more biologically active T_3 (3′,3,5-L-triiodothyronine) is produced peripherally via an outer-ring deiodination (ORD) reaction. Three deiodinases have been identified in vertebrates. Deiodinase type 1 (D1) has both ORD activity (converts T_4 to T_3) and inner ring deiodinase (IRD) activity (converts T_4 and T_3 to less active metabolites). Deiodinase type 2 (D2) has only ORD (activation) activity, and deiodinase type 3 (D3) has only IRD (inactivation) activity [47]. Thus, TH deiodinases are key regulators of TH action by selectively removing iodide molecules, dictating their relative biological activity [3,16].

Abbreviations: THs, thyroid hormones; D2, deiodinase type 2; D3, deiodinase type 3; SECIS, selenocysteine insertion sequence; IRD, inner ring deiodinase; ORD, outer ring deiodinase; CF, condition factor; TRs, thyroid hormone receptors; UPM, universal primer mix; NUP, nested universal primer.

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The critical role of deiodinases in regulating differential timing of tissue morphogenesis is well established in anuran models [2,16]. Likewise, distinct temporal and spatial patterns of deiodinase gene expression or activity are observed during the development of a variety of fishes including zebrafish, Atlantic salmon, rainbow trout, and Senegalese sole [20,30]. Tissue-specific regulation of TH levels by deiodinases has been suggested as a mechanism to account for the differences in lamprey serum TH profiles compared to other metamorphosing vertebrates [12,13].

The intestine is the primary site of deiodination in lampreys and intestinal deiodinase activity fluctuates in concert with serum TH levels during the lifecycle [12]. *P. marinus* T_4ORD activity in the intestine and liver is highest prior to and during the early stages of metamorphosis, when serum T_3 levels are also elevated. Intestinal and hepatic IRD activity predominates in the later stages, when serum T_3 levels are also lower [12,13]. Given the correlation between lamprey serum T_3 levels and deiodinase activities, and the importance of peripheral deiodination in mediating circulating TH concentrations and tissue morphogenesis in other species, we sought to isolate the D2 cDNA from *P. marinus* and examine its expression profile (mRNA levels) in terms of tissue-specificity, developmental regulation, and response to thyroid challenges.

2. Materials and methods

2.1. Animal collection and maintenance

Larval *P. marinus* were collected from Oshawa Creek, Ontario, Canada and the Sturgeon River near Wolverine, Michigan, United States using backpack electrofishers. The larvae were transported to the aquatics facility at the University of Regina, where they were housed and permitted to complete metamorphosis as previously described [26]. The larval period of the lifecycle consists of a growth phase, an immediately premetamorphic phase (i.e., CF) and the phase of metamorphosis. Immediately premetamorphic larvae are >120 mm in length, >3 g in weight and have a condition factor (CF) of >1.5 (CF = weight (g)/(length (mm))$^3 \times 10^6$ [43]) and are referred to as "CF larvae". Staging (stages 1–7) of lamprey metamorphosis is based on external morphology and the developmental changes in the eyes, oral apparatus, branchiopores, and colouration [42].

Prior to processing, all lampreys were anaesthetized in 0.05% buffered tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) and euthanized by decapitation. Brain, heart, liver, intestine, and kidney tissues were harvested from small, non-CF larvae (<120 mm and <3.0 g), CF larvae, larvae in each of the seven stages of metamorphosis, and from parasitically feeding juveniles. Following removal, tissues were flash-frozen in liquid nitrogen and stored at −86 °C. All animal handling and procedures were approved by the President's Committee on Animal Care at the University of Regina and were consistent with the guidelines of the Canadian Council on Animal Care. Total RNA was isolated from frozen tissues using Trizol reagent (Invitrogen Life Technologies, ON, Canada) according to the manufacturer's protocol and stored at −86 °C in diethylpyrocarbonate (DEPC)-treated water. Messenger RNA (mRNA) was purified from total RNA using the Ambion Poly(A)Purist MAG Kit (Invitrogen Life Technologies).

2.2. Cloning of P. marinus D2

Approaches used to obtain the full-length coding region of the *P. marinus* D2 mRNA included semi-degenerate PCR (polymerase chain reaction) and numerous variations of 5′ and 3′ RACE (Random Amplification of cDNA Ends). Semi-degenerate PCR primers (DD2F2 and DD2R2; Table 1) were designed using the CODEHOP program [32], a multiple sequence alignment of the D2 amino acid sequences from several vertebrates and the *P. marinus* codon usage table. All primers were synthesized by Sigma Genosys (Mississauga, ON, Canada). For semi-degenerate PCR, reverse transcription was performed with the Fermentas RevertAid M-MuLV reverse transcriptase kit (Fermentas, ON, Canada) using 6 μg total RNA isolated from larval intestine and 25 pmol of DD2R2 primer (Table 1). PCR was carried out using the aforementioned cDNA, DD2F2 and DD2R2 primers and Taq polymerase (Fermentas). A 119 bp PCR product was excised from a 3% agarose gel, inserted into the TOPO pCRII vector (Invitrogen Life Technologies) and sequenced by DNA Landmarks, Quebec, Canada. Gene-specific primers for RACE (Table 1) were designed using the 119 bp *P. marinus* D2 product and the Primer 3 program [33]. 5′ and 3′ RACE reactions were performed under various conditions, using the primers listed in Table 1 and reagents from several manufacturers. Details are reported in the supplementary material.

2.3. Northern blotting to determine P. marinus D2 transcript size

Northern blotting was performed as previously described [26]. Briefly, a Northern blot containing five micrograms of total RNA from small non-CF larval lampreys (intestine, liver, and kidney) was probed with a 330 bp fragment of D2 cDNA labelled with ^{32}P-dCTP. Northern blots were washed, exposed to phosphor screens and visualized on a Storm 860 Imager (Amersham Biosciences, QC, Canada) [26].

2.4. Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR was used to examine the tissue-specific developmental changes in D2 mRNA levels in the intestine, liver, kidney, and brain, following the guidelines recommended by Bustin et al. [8]. For each tissue developmental series, the levels of D2 mRNA (quantified in triplicate for each of 5 biological replicates) were normalized against the β-actin mRNA levels (BA; quantified in triplicate for the same 5 biological replicates). D2 was amplified using primers D2-1049-S and D2-1138-AS and BA was amplified using primers BACT#4_S_FL1119 and BA-1371R-S2 (Table 1). RNA quality was confirmed using denaturing agarose gel electrophoresis and purity was confirmed using spectrophotometry ($A_{260}/A_{280} > 1.85$). One microgram of RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, ON, Canada) according to the manufacturer's protocol and stored at −86 °C. In all instances, 'no-RT controls' and intron spanning primers confirmed there was no genomic DNA contamination. The QuantiTect™ SYBR Green real-time PCR kit (Qiagen) was used as per manufacturer's instructions.

Real-time PCR was performed using an iQ5 Thermocycler (Bio-Rad Laboratories, ON, Canada) and the following parameters: 95 °C × 15 min; 45 cycles of 95 °C × 15 s, 60 °C × 30 s, 72 °C × 30 s. The iQ5 Real-Time PCR Detection System and software were used to determine the normalized levels of D2 mRNA relative to BA. In all cases, the default quantification cycle (C_q; also described as threshold cycle (C_t)) values were used. Real-time reactions were free of spurious amplicons, as determined through amplification-free water controls and melt-curve analysis (amplification at 10 s/interval from 55–95 °C in 0.5 °C intervals).

Serial dilutions of pooled cDNA for each tissue (2–0.0001 μl of RT reaction) were used to generate triplicate standard curves encompassing the dynamic range for each gene-tissue combination. During the validation phase, these dilutions were used to optimize the PCR, establish the dynamic range and efficiency, and determine the appropriate amount of starting template for each gene-tissue combination (Supplementary Table 1) [8]. Later,