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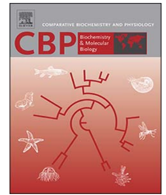

Genome specific *PPARαB* duplicates in salmonids and insights into estrogenic regulation in brown trout

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are key regulators of many processes in vertebrates, such as carbohydrate and lipid metabolism. PPARα, a member of the PPAR nuclear receptor gene subfamily (NR1C1), is involved in fatty acid metabolism, namely in peroxisomal β-oxidation. Two gene paralogues, *pparαA* and *pparαB*, were described in several teleost species with their origin dating back to the teleost-specific genome duplication (3R). Given the additional salmonid-specific genome duplication (4R), four genes could be theoretically anticipated for this gene subfamily. In this work, we examined the *pparα* gene repertoire in brown trout, *Salmo trutta* f. *fario*. Data disclosed two *pparα*-like sequences in brown trout. Phylogenetic analyses further revealed that the isolated genes are most likely genome *pparαB* duplicates, *pparαBa* and *pparαBb*, while *pparαA* is apparently absent in salmonids. Both genes showed a ubiquitous mRNA expression across a panel of 11 different organs. *In vitro* exposed primary brown trout hepatocytes strongly suggest that *pparα* gene paralogues are differently regulated by ethinylestradiol (EE2). *PparαBb* mRNA expression significantly decreased with dosage, reaching significance after exposure to 50 μM EE2, while *pparαBa* mRNA increased, significant at 1 μM EE2. The present data enhances the understanding of *pparα* function and evolution in teleost, and reinforces the evidence of a potential crosstalk between estrogenic and *pparα* signaling pathways.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) (NR1C) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily (Issemann and Green, 1990). Activated PPARs heterodimerize with the retinoid X receptor (RXR) by binding to specific upstream recognition sites, the peroxisome proliferator response elements (PPREs), located in the promoter regions of target genes (Issemann et al., 1993; Tugwood et al., 1992). The ligand-binding domain (LBD) of the receptor changes and triggers the release of corepressor proteins and the recruitment of coactivator proteins, which ultimately promote the transcription of the target genes (Xu et al., 1999).

In vertebrates, the PPAR gene subfamily consists of PPARα, PPARγ and PPARβ (also known as PPARδ), which were identified in different mammalian species (Aperlo et al., 1995; Issemann and Green, 1990; Ngo et al., 2007; Sher et al., 1993; Tugwood et al., 1998), birds (Diot and Douaire, 1999), frogs (Dreyer et al., 1992) and fish (Andersen et al., 2000; Boukouvala et al., 2004; Leaver et al., 2005, 2007; Robinson-Rechavi et al., 2001; Ruyter et al., 1997). PPARα, β and γ have different physiological functions and recognize different exogenous and endogenous ligands. Regarding PPARα, exogenous ligands include primarily hypolipidemic drugs, while a variety of saturated and unsaturated fatty acids are considered endogenous ligands (Corton et al., 2000). PPARα is involved in lipid oxidation through the regulation of the expression of several genes directly involved in peroxisomal β-oxidation (Lee et al., 1995). PPARα has been associated with the peroxisome proliferation phenomenon in rodents caused by certain chemicals, the so-called

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peroxisome proliferator (PP) compounds, consisting of an exacerbated increase of peroxisome number, peroxisomal enzyme activities (Hess et al., 1965; Nemali et al., 1989), and ultimately hepatocarcinogenesis (Reddy et al., 1983). The characteristic peroxisomal morphological and biochemical changes provoked by PP administration are absent in PPARα-null mice, proving the importance of this receptor on this process (Lee et al., 1995; Peters et al., 1997).

Whole-genome duplications (WGD) are specific polyploidization events, in which occurs the doubling of all gene repertoire of an organism (Ohno, 1970). These events are of extreme importance to understand eukaryote evolution, contributing with new genetic material to species diversification (Crow and Wagner, 2006; Hultqvist et al., 2012; Ohno, 1970). Vertebrate evolution has been marked by various WGD events. The first two rounds (2R) are proposed to have occurred at the base of vertebrate radiation (Dehal and Boore, 2005; Putnam et al., 2008), although the exact timing is still a matter of discussion (Smith and Keinath, 2015), with a third round (3R) dating to the origin of the teleosts (Jaillon et al., 2004). Recently, a fourth round (4R) was undoubtedly identified immediately before the rising of salmonids around 96 million years ago (Berthelot et al., 2014; Lien et al., 2016; Rondeau et al., 2014). The 3R genome duplication has clearly impacted the evolution of the nuclear receptor gene repertoire; in the case of *pparα*, two genes, *pparαA* and *pparαB*, are present in teleost species such as zebrafish and pufferfish (Bertrand et al., 2007). Thus, following the salmonid specific genome duplication, the emergence of four isoforms of the *pparα* could be theoretically anticipated. The full genome sequence currently available of evolutionary informative species such as spotted gar, northern pike and atlantic salmon (Braasch et al. 2016; Lien et al., 2016; Rondeau et al., 2014) provides a valuable tool to infer gene family history in Actinopterygii. For example, in the case of *ppar*'s, the impact of 4R was determined with the identification of four *pparβ* gene paralogues in Atlantic salmon (Leaver et al., 2007). In contrast, a partial sequence of a single *pparα* gene has been isolated and characterized so far in salmonid species (Batista-Pinto et al., 2005).

The main aim of this study was to investigate the *pparα* gene repertoire in the brown trout, *Salmo trutta* f. *fario*, as a representative species of *Salmonidae* family. We isolated two full *pparα* sequences and examined their phylogenetic relationship to known *pparα* isoforms from other teleost species. In order to get insights into potential different functions, their organ distribution and their regulation by estrogenic stimuli were studied in the context of a previously reported crosstalk of estrogens and PPARs (Rocha et al. 1999, Batista-Pinto et al. 2009).

2. Materials and methods

2.1. Animals

Brown trout (*Salmo trutta* f. *fario*) specimens were obtained from a national aquaculture facility (Aquaculture Station of Torno, Amarante, Portugal). Two adult mature animals of both sexes, approximately 2 years-old, were used for organ collection. Females and males weighed 294.0 ± 19.5 g and 369.8 ± 57.7 g, and had a total length of 29.3 ± 1.8 cm and 30.4 ± 1.7 cm, respectively; values are given as mean ± SD. For the isolation of primary hepatocytes, we used two juvenile individuals of about one year-old, with a mean weight of 79.0 ± 2.5 g and total length of 19.0 ± 0.0 cm. All animals were maintained at 16 °C under a natural photoperiod. Water quality was ensured throughout all the acclimatization period prior to experiments (minimum four weeks). Animals were fed 3 to 4 times per week with dry granules for salmonids (T-4 Optiline, Skretting) and fasted for at least 24 h before the experiments. All animal handling followed the Portuguese Decree-Law No. 113/2013 implementing EU Directive No. 2010/63 on animal protection for scientific purposes.

2.2. Isolation of primary hepatocytes and culture conditions

Fish were euthanized by immersion in an aqueous solution of ethylene glycol monophenyl ether (0.6 mL/L) (Merck), and the primary hepatocytes isolated according to a two-step collagenase perfusion technique, previously described in detail (Madureira et al., 2015). Hepatocytes were plated at 1×10^6 cells/mL in 500 μL of Leibovitz's L-15 medium without phenol red (Invitrogen), with 5% charcoal stripped fetal bovine serum (FBS) (Sigma-Aldrich), 100 μg/mL of streptomycin and 100 U/mL of penicillin (PAA Laboratories GmbH). Cells were maintained at 19 °C and exposures started after 24 h for the duration of 72 h, with daily changes of solutions. Treatment conditions included: 0.1% of ethanol p.a (Merck) – solvent control (SoCo), 1, 10 and 50 μM of EE2 (CAS 57-63-6, Sigma-Aldrich). Each group was tested in five biological replicates (hepatocytes derived from two individual fish), distributed in three different culture plates. Cell viability (assessed by the trypan blue exclusion assay) was always > 94.6%.

2.3. Cloning of pparαBa and pparαBb isoforms

New sequences of the *pparαBa* and *pparαBb* genes in brown trout were obtained through a degenerate RT-PCR strategy and RACE-PCR (Table 1). RACE-primers for *pparα* were designed on a partial known sequence of brown trout (NCBI DQ139936), which was later recognized as the *pparαBb* isoform. For determination if other *pparα* isoforms were present in brown trout, degenerate primers were designed based on *pparα* sequences from other teleost species obtained from databases (Ensemble, NCBI). Block Maker (Henikoff et al., 1995) and CODEHOP software (Rose et al., 1998) were used to find conservative sequences on the aligned input sequences and to select degenerated primers. Advantage 2 polymerase (Advantage 2 PCR kit, Clontech) was used to amplify sequences. The 5'RACE and 3'RACE cDNA were prepared from brown trout liver for sequence elongation according to the manufacturer's instructions (SMARTer RACE cDNA amplification kit, Clontech, USA). Two μL of a 1:10 diluted cDNA was used in PCR reactions with the following cycle conditions: degenerate primer PCR, 30 s at 95 °C,

Table 1
Overview of the primer sequences used for cloning of *pparαBa* and *pparαBb* in brown trout (*Salmo trutta* f. *fario*).

Primer name	PCR strategy	Primer sequence, forward, 5′–3′	Primer sequence, reverse, 5′–3′	Sequence lenght
pparαBa				
elongation to 5′-end	5′-RACE	universal primer, Clontech	CACGTTCACCAGGCCAGGGCGGTCTC	
	5′-nested RACE	universal primer, Clontech	GCAGATGATGGCAGCCACAAACAGAG	1347 bp
elongation to 3′-end	3′-RACE	GAGGCACTGTTCGCCCTGCTGGCATC	universal primer, Clontech	
	3′- nested RACE	ATCGGGCTTCATCACCAGGGAGTTC	universal primer, Clontech	2227 bp
pparαBb				
first part of sequence	degenerate primers	ACCGTGGGAATGTCCCAYAAYGCNAT	CCATGGAGTTGAATCTCGTAGCRAAYTGRAA	600 bp
elongation to 5′-end	5′-RACE	universal primer, Clontech	GTAGGTCAGGTCCAGGCGTGAGAAG	
	5′-nested RACE	universal primer, Clontech	GGACAGACTAGGCGAACTCAGTCAG	1112 bp
elongation to 3′-end	3′-RACE	GGCCAGGCACATCTATGAGGCCTAC	universal primer, Clontech	
	3′- nested RACE	CCCACAGACCATCACTCCACCATTTG	universal primer, Clontech	1573 bp

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