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Short communication

Indoleamine 2,3-dioxygenase: First evidence of expression in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The role of enzymes as active antimicrobial agents of the innate immunity in teleost fish is proposed in diverse works. Secretion of Indoleamine 2,3-dioxygenase (IDO) has been described in higher vertebrates; it degrades L-tryptophan in extracellular environments associated mainly with mucosal organs. The effect of IDO on decreasing amino acid concentration may inhibit the growth of potential pathogens. In fish the study of this molecule is still. Here we report the identification of an *Onchorhyncus mykiss* IDO homologue (*Om*IDO). IDO was cloned, sequenced, and the primary structure shows conservation of key functional sites. The constitutive expression is altered when the fish is challenged with LPS as a pathogen-associated molecular pattern (PAMPs). Up-regulation of IDO was shown preferentially in the fish's mucosal cells. In order to obtain evidence of a possible regulation mechanism, an *in vitro* cell model was used for to show that *Om*IDO is induced by rIFN. These study has identified a Indoleamine 2,3-dyoxigenase in *O. mykiss* will contribute to expands our knowledge of the function this protein in fish immune response. These findings allow to propose the use of OmIDO as a molecular indicator of strength of the animal's immune response and wellbeing.

1. Introduction

L-Trp is an essential amino acid and a precursor of bioactive compounds such as the neurotransmitter serotonin and the hormone melatonin (Sainio et al., 1996). In teleost fish, just as with higher vertebrates, it has been shown that the biochemical pathways for generating these molecules are conserved, with high levels of expression of homologous hydrolase enzymes in the nervous system and the pineal gland of the fish (Bégay et al., 1998; Coon et al., 1998).

It has been stated that the cells that participate in inflammatory responses break down tryptophan along the kynurerine path (Moffett and Namboodiri, 2003). As a result, in recent years several studies have been conducted to analyse the relationship between tryptophan catabolism and the generation of immune response.

In mammals, the first stage of the kynurenine pathway is the oxygenative decyclization of L-tryptophan to form *N*-formylkynurenine, which is catalysed by the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) and the extrahepatic enzyme indoleamine 2,3-dioxygenase (IDO). The latter is induced by bacterial Lipopolysaccharide (LPS) (Yoshida and Hayaishi, 1978) and is therefore directly related to inflammatory processes. IDO is constitutively expressed in the lung, gut, spleen, liver, kidney, stomach and brain (King and Thomas, 2007). It is formed by two alpha-helical domains with a prosthetic HEME group positioned between these two structures. It was isolated and identified for the first time in 1967 from rabbit gut and its name derives from its use of Indol-derived compounds as substrates (Yamamoto and Hayaishi, 1967; Yuasa et al., 2009).

Higher vertebrates possess two IDO enzymes: IDO1 and the recently reported IDO2 (Ball et al., 2007; Metz et al., 2007). Other related molecules have been described from studies into the primary structure and enzymatic activity of the molecule (Yuasa et al., 2007).

The property that IDO can be induced by pathogen-associated molecular patterns (PAMPs) was described by Yoshida et al. who

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reported that the overexpression of IDO by LPS and poly I:C is dependent on interferon-gamma (IFN-γ) (Yoshida et al., 1986). In mammals, the induction of the enzyme by IFN-γ has been described in dentritic cells, monocytes, macrophages, eosinophils, epithelial cells, fibroblasts, smooth muscle, and endothelial cells (King and Thomas, 2007). IDO is strongly induced by IFN-γ but poorly induced by IFN α and β. It is therefore considered a differential molecular marker for type-I and II interferons in higher vertebrates (Byrne et al., 1986). IFN-γ is a key component of the innate and adaptive immune response for intracellular virus and bacteria control (Schoenborn and Wilson, 2007). We may therefore speculate that the connection between the metabolism of tryptophan and the immune system is through decreased availability of the amino acid for intracellular pathogens, thus impairing proliferation of pathogens (Moffett and Namboodiri, 2003).

Some years ago, it was thought that this enzyme was present only in higher vertebrates. However, Yuasa et al. (2007) described IDO2 in the genome of zebra fish (*Danio rerio*). Functional analysis showed that the molecule presented a K_m for L-TRP approximately 500–1000 times higher than the mammal isoform and it was assumed that IDO1 probably evolved from IDO2, improving its specificity for tryptophan (Yuasa et al., 2007). In teleosts, it was been described that IFN-γ is functionally similar to that of mammals, as it conserved the JAK-STAT signalling pathways, which are induced by this cytokine (Zou et al., 2005). Genomic and proteomic analysis of the RTS-11 and SHK-1 cell lines revealed some of the genes that are regulated by IFN-γ in rainbow trout and Atlantic salmon. These studies describe how stimulation with IFN-γ increases the transcription levels of immune response regulator genes in fish, with the presence of antigens and antimicrobial peptides (Martin et al., 2007). However, the expression of IDO by induction with IFN-gamma has not been previously described.

The present study describes and characterises a coding sequence of IDO for rainbow trout (*Oncorhyncus mykiss*), namely *Om*IDO, while also demonstrating its constitutive expression in different organs. Performing *in vivo* assays it was also shown that *Om*IDO is up-regulated by LPS, especially in mucosal organs, and most notably in gills. Finally, with the aim of proving a probable immune regulation mechanism, the up-regulation of the enzyme by rIFN-γ was also shown on the RTS-11 cell line.

2. Materials and methods

2.1. Animals

Twelve Rainbow trout (100–200 g) were purchased and maintained in 1-m-diameter fiberglass tanks supplied with recirculating freshwater at 9–12 °C (Laboratory of Applied Genetics-Pontificia Universidad Catolica de Valparaíso, CHILE). The fish were acclimatized for two weeks before experimental manipulation and fed twice daily with a commercial pellet trout diet.

2.2. Bioinformatics analysis

The amino acid sequence of *Om*IDO was used to perform phylogenetic analysis. Accession numbers of the sequences used for homology comparisons and phylogenetic analysis are listed in S1 table. The trees were constructed using the software MEGA version 6.0 (Tamura et al., 2013). Additionally, the three-dimensional structure of *Oncorhyncus mykiss* *Om*IDO was modelled using the I-TASSER modelling server (Yang et al., 2014), for which human indoleamine 2,3-dioxygenase (PDB ID: 2D0T) was used as the main template. The model was then analysed with MacPymol software.

2.3. Fish induction and tissue collection

Fish were separated as control and LPS groups. The fish were anaesthetized with benzocaine (25 μg/L) prior to treatment and challenged by intraperitoneal injection with 1 mg of *E. coli* LPS (Sigma-Aldrich, UK) or phosphate buffered saline (PBS [control]). The animals were euthanised 12 h after challenging by immersion in a solution of 50 mg/L benzocaine. The tissues (head kidney, spleen, gut, skin, liver, gill and brain) were collected for RNA extraction using RNAlater solution (Ambion, USA) in accordance with the manufacturer's instructions. This study was carried out in accordance with Law 20,380 regarding animal welfare, as set out by the Chilean Health Ministry in the use of wild or protected animal species in biomedical research and approved by the Pontificia Universidad Catolica de Valparaíso Bioethical Committee.

2.4. Cloning and rIFN-γ expression

The coding sequence for IFN-γ for salmonids was obtained from the GenBank database (Access number: NP_001117030.1) and was cloned in the pJexpress 401 vector (DNA 2.0©, Inc.). The protein was induced by addition of 1 mM IPTG, followed by culture for 6 h at 37 °C. The rIFN-γ was purified under denaturing conditions on a pH gradient using Ni^{2+}nitryloacetic column (Ni-NTA) (Qiagen, Germany). Endotoxins were removed by affinity chromatography with Polymyxin B-Agarose (Sigma-Aldrich, UK) in accordance with the manufacturer's instructions. The integrity and purity of the IFN-γ was analysed by SDS-PAGE and MALDI-TOF mass spectroscopy.

2.5. Cell culture and induced expression in vitro

RTS-11 cells were cultivated in 75 cm^2 flasks containing complete Leibovitz (L-15) medium (GIBCO, UK) supplemented with 10% foetal calf serum (FCS) (Biological Industries, Israel) and penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively) (Biological Industries, Israel) and incubated at 20 °C. The cells were seeded at a density of 9×10^5/well into 6-well plates and stimulated with rIFN-γ (10 ng/mL) (Zou et al., 2005) or the medium alone as negative control. At times 2 h, 4 h, 8 h, 12 h, 24 h and 48 h the cells were dissolved in TRIzol® reagent (Life Technologies, USA) for RNA extraction.

2.6. Quantitative evaluation of mRNA levels IDO by RT-PCR

For the gene expression quantification, 1 μg RNA from each sample was pre-treated with 2 units of DNase RQ1 (Promega, Germany) and incubated at 37 °C for 20 min. The cDNA synthesis was performed using AffinityScript cDNA Synthesis Kit (Agilent Technologies, USA) in accordance with manufacturer's instructions. Specific primers were used to amplify the *Om*IDO and the endogenous control ELF-1 transcripts (S2 Table). Real-time PCR was performed using 15 μl reaction mixtures containing: Brilliant II SYBR®Green QPCR MM (Agilent Technologies, USA), 1 μM of each primer, and 2.5 μl of cDNA in Mx3000P qPCR System (Agilent Technologies, USA). The qPCR was performed in duplicate per tissue sample or n = 6 in the case of the expression *in vitro*, including an initial denaturation step of 3 min at 94 °C followed by amplification of the target cDNA (35 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s and the extension time at 72 °C for 15 s) and fluorescence detection. The relative expression of IDO mRNA in tissues was calculated as arbitrary units and normalized with the expression level of rainbow trout ELF-1 housekeeping gen. For the cell samples the fold change was calculated according to the formulae $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).