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Role of Prostaglandins in the Immune System of Gilthead Seabream (*Sparus aurata* L.)

Papel de las Prostaglandinas en el Sistema Inmunitario de Dorada (*Sparus aurata* L.)

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5-HPETE	5-hydroxyperoxy-eicosatetraenoic acid
5-LO	5-lipooxygenase enzyme
15-HPETE	15-hydroxyperoxy-eicosatetraenoic acid
15dPGJ2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
15-LO	15-lipoxygenase enzime
AA	arachidonic acid
Ab	antibody
AC	adenylate cyclase
actb	β-actin gene
AGs	acidophilic granulocytes
arg	arginase
ATP	adenosine triphosphate
BADGE	Bisphenol A Diglycidyl Ether
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	central nervous system
COX	cyclooxygenase
cPGE	cytosolic PGES
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CRTH2	chemoattractant receptor homologous molecule expressed by T helper 2 cells
CyPG	cyclopentenone prostaglandin
DAMP	damage-associated molecular pattern
dbcAMP	29-dibutyryladenosine 39,59-cyclic monophosphate sodium salt
DHA	docosahexaenoic acid
diMePGE$_2$	16,16-dimethyl-PGE$_2$
DMSO	dimethyl sulfoxide
DNA	desoxyrribonucleic acid
DNase	deoxyribonuclease
DP1	prostaglandin D2 receptor 1
DP2	prostaglandin D2 receptor 2
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EP	prostaglandin E_2 receptor
EPA	eicosapentaenoic acid
F	forward oligonucleotide

FBS	fetal bovine serum
FCS	fetal calf serum
FP	prostaglandin $F_{2\alpha}$ receptor
G7+	acidophilic granulocyte-enriched cell suspension/fraction
G7-	acidophilic granulocyte-lacking cell suspension
GPR	G protein-coupled receptor
HK	head kidney
HPGD	15 hydroxy-prostaglandin-dehydrogenase
H-PGDS	hematopoietic prostaglandin D synthase
HPLC	high-performance liquid chromatography
IFNγ	gamma interferon
Ig	immunoglobulin
IL-1β	interleukin 1-beta
IL-6	interleukin-6
IL-10	interleukin-10
IP	prostaglandin I_s receptor
L-PGDS	lipocalin prostaglandin D synthase
LP	lipoxin
LPS	lipopolysaccharide
LT	leukotrienes
MACS	magnetic-activated cell sorting
MAF	macrophage-activating factors
MC	mast cell
MHCI	major histocompatibility complex I
MHCII	major histocompatibility complex II
mPGES	membrane associated prostaglandin E synthase
mrc1	mannose receptor c-type 1
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NLR	nucleotide-binding oligomerization domain (NOD)-like receptors
NK	natural killer cell
NOS	nitric oxide syntase
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PC	prostacyclins
PE	peritoneal exudates
PFC	plaque-forming cell

PFOA	perfluorooctanoic acid
PG	prostaglandin
PGDS	prostaglandin D synthase
PGES	prostaglandin E synthase
PGN	peptidoglycan
PI	propidium iodide
PKA	protein kinase A
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
POA	palmitoleic acid
PPAR	peroxisome proliferator activated receptor
PRR	pattern recognition receptor
PPRE	peroxisome proliferator responsive elements
PTGS2	prostaglandin-endoperoxide synthase 2
R	reverse oligonucleotide
RLR	retinoic acid-inducible gene (RIG)-I-like receptors
RNA	ribonucleic acid
RNS	reactive nitric species
ROS	reactive oxygen species
RXR	retinoid X receptor
rps18	ribosomal protein S18 gene
SHK-1	salmon head kidney cell line 1
sRPMI	RPMI-1640 culture medium supplemented with 0.35% NaCl
Tc	T cytotoxic cell
Th	T helper cell
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TP	thromboxane A_2 receptor
TX	thromboxanes
VaDNA	genomic DNA from *Vibrio anguillarum*
VaLPS	lipopolysaccharide from *Vibrio anguillarum*

SUMMARY

The immune system of teleost fish has been widely studied for decades but, much is still unknown, especially concerning the role of prostaglandins (PGs) and their signaling pathways. The gilthead seabream (Sparus aurata L.), is a marine teleost fish widely used as a research animal model for better understanding the fish immune response. Despite this, few studies have been focused on the role of PGs in gilthead seabream phagocytes. Within this framework, the aim of this thesis is to advance our knowledge of the role of PGs in the immune response of the gilthead seabream.

In the first chapter, we study the molecular characterization of two genes (*pgds1* and *pgds2*) which deduced amino acid sequences show high degree of similarity to mammalian Lipocalin-PGDS involved in PGD_2 synthesis. Tissues samples of seabream juveniles challenged or not with the pathogen *Vibrio anguillarum* or AGs stimulated with PAMPs were analyzed by RT-qPCR. The results showed that both genes were expressed in all tissues and their expression were modulated upon bacterial challenge in almost all tissues examined. However, *pgds1*, but not *pgds2*, was detected in AGs which expression was upregulated by stimulation with PAMPs with the same kinetics than PGD_2 by this cell type, as was determined by enzyme immunoassay. Finally, to determine the effects of PGD_2 in the main functions of AGs, control or stimulated AGs were cultured with PGD_2. The results showed that treatment of AGs with PGD_2 results in decreased ROS production and cytokine expression. These results provide us evidences that PGD_2, probably synthesized by PGDS1, could play a key role in the resolution of inflammation by AGs in gilthead seabream.

In the second chapter, we analyzed by RT-qPCR the expression profile of genes involved on PGE_2 metabolism in tissue samples of seabream juveniles challenged or not with *Vibrio anguillarum* and phagocytes activated with genomic DNA from *V. anguillarum* (VaDNA) were analyzed by RT-qPCR. The results showed that *pges* (involved in PGE_2 synthesis) was expressed in all tissues examined as well as in phagocytes and its expression was up-regulated in all immune organs upon bacterial challenge and in phagocytes stimulated with VaDNA. In contrast, the mRNA levels of *hpgd* (involved in PGE_2 catabolism) were lower in all tissues tested from challenged fishes. In addition, the effect of PGE_2 in the main functions of AGs was analyzed. Treatment of stimulated AGs with PGE_2 resulted in decreased ROS production and cytokine expression. In contrast, treatment of stimulated macrophage with PGE_2 resulted in increased mRNA levels of *il10*, *mrc1* and *arg2* and decreased *il6* ones, resulting in a M2 phenotype. Furthermore, PGE_2 signaled through the cAMP/PKA/CREB pathway in seabream macrophages. These results points to PGE_2 could play a key role in the regulation of inflammation in gilthead seabream.

Finally, in the third chapter, we determine the effects of 15deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$) and its signaling pathway in seabream AGs. $15dPGJ_2$ is a cyclopentenone PG derivated from PGD_2 that can signal through PPARγ, DP2 receptor or can act by covalent binding to proteins. For this purpose, seabream AGs were stimulated with VaDNA and treated with $15dPGJ_2$, with $15dPGJ_2$ analogs, and with

PPARγ agonists and antagonist. The results showed that $15dPGJ_2$ decreased ROS production and cytokine expression in stimulated AGs. Similarly, PG analogs and PPARγ agonist were able to mimic the effects of $15dPGJ_2$ in ROS production by stimulated AGs. Besides, those effects were reversed by PPARγ antagonists. These results show that, $15dPGJ_2$ could have an anti-inflammatory role in the resolution of the inflammation in gilthead seabream which signal both, through protein covalent binding and PPARγ activation.

INTRODUCTION

0. Overview

In a world inhabited by more than 7 million people and where will be occupied by more than 9 million people by 2050, the greatest challenge to face is how to feed the world population and, at the same time, how to deal with the effects of the climate change.

In 2015, the 17% of the world's animal protein intake derived from fish and accounted for 6.5% of all protein consumed, meaning the 20% of the total protein intake *per capita* in developing countries. Moreover, in 2016, the 47% of global fish production came from the aquaculture and the 88% was set aside for the human consumption (FAO, 2018). In 2016, Spain was the main aquaculture producers of the Europe Union (EU) being the EU the most important world market to the aquaculture products (APROMAR, 2018). Besides, Region of Murcia is the first producer of sea bass and the second ones of gilthead seabream in Spain (APROMAR, 2018). This data, together with the fact that fishing production does not increase while aquaculture has experienced a marked increase since 2000, both, around the world and in Spain, point out the relevance of the aquaculture in the present society.

Aquaculture consist in the aquatic production of animals and plants using different methods and techniques to improve its efficiency. This means, individuals are exposed to handling in a limited space triggering stress in animals which causes the development of infectious diseases that are responsible for high mortality and substantial economic losses. So the study of fish immune response would be redundant in higher efficiency and decreased economic losses in aquaculture. Moreover, fish occupy a key position from the evolutionary point of view due to they are the first group of animals where is present the innate immune response and the adaptive ones based on the rag system. Consequently, the knowledge of fish immune system has become one of the main objective in aquaculture research.

The gilthead seabream (*Sparus aurata* L.) is a marine, seasonally breeding, protandrous hermaphrodite teleost fish. Although this fish species has been used as a research animal model to study the fish immune system, much is still unknown. In this context, prostaglandins (PGs) are lipid mediators with a key role in the modulation of the immune function, triggering pro-inflammatory or anti-inflammatory response during the inflammation process. They are produced by the sequential metabolism of araquidonic acid (AA) of the cell membrane by the action of the enzyme phospholipase A_2 (PLA_2). In mammals, PGE_2 and 15deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$) are the main studied. They exert their biological actions through the binding to G protein-coupled receptors (GPRs) and peroxisome proliferator receptor gamma (PPARγ), respectively. However, little is known about the synthesis, the role of different PGs in fish immune response and the signaling pathways involved in their actions.

This PhD thesis has been developed in the research group *Innate Immune System of Teleost Fish*, that have so many years of experience in the study of the immune system of aquaculture species

with a great economic interest, such as, sea bream (*Sparus aurata* L.) and sea bass (*Dicentrarchus labrax* L.).

Located in the above framework, this thesis has been focused on the role of PGs in fish immunity using the gilthead seabream as a research model. Specifically, it has been studied the role of different PGs (PGE_2, PGD_2, synthetic PGD_2 analog, $15dPGJ_2$, synthetic $15dPGJ_2$ analog and Δ^{12}-PGJ_2) in the main biological functions of phagocytic cells (macrophages and acidophilic granulocytes), the molecular characterization of prostaglandin D synthase (PGDS), the expression profile of different genes involved in prostaglandin metabolism in vitro and in vivo and the signaling pathways involved in PGs actions.

1. Immune system

The main function of the immune system is the defense of the organism against foreign substances including microorganisms, such as viruses, bacteria, fungi, protozoa and multicellular parasites, and macromolecules, such as proteins and polysaccharides (González and Larrea 2010; Abbas et al. 2015). This system is able to distinguish between self and non-self molecules or cells, and usually react only to non-self components (Kindt et al. 2007; Male et al. 2007). The immune system is classically divided into two types: i) innate, natural or non-specific, and ii) adaptive, acquired or specific.

The innate immune response, which is the oldest defense system from a phylogenetic point of view, is present in 98% of the pluricellular organisms, while adaptive immunity appeared later in more evolutionary advanced animal species. Innate immunity acts as the first response against infection until the adaptive response is triggered. The biochemical and cellular mechanisms of the innate response are present even before the organism is exposed to the pathogen and it acts in the same way each time that the infection appears. However, the adaptive immune response is highly specific for a particular pathogen and becomes more effective with each successive encounter with the same pathogen.

The main components of the innate immune system are: i) physical and chemical barriers, ii) phagocytic cells (neutrophils and macrophages), eosinophils and natural killer cells (NKs), iii) blood proteins such as complement and acute phase proteins, and iv) cytokines (Abbas et al. 2015). The innate immune mechanisms are specific for common structures in microbial groups but are unable to distinguish between small differences in exogenous pattern recognition receptors (PRRs). Those mechanisms include Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors, nucleotide-binding oligomerization domain-like receptors, and C-type lectin receptors (Takeuchi and Akira 2010). The PRRs recognize pathogen-associated molecular patterns (PAMPs) conserved in pathogenic organisms such as polysaccharides, lipopolysaccharides (LPS), bacterial DNA or viral RNA.

These receptors also recognize endogenous molecules released from damaged cells, known as damage-associated molecular patterns (DAMPs) (Takeuchi and Akira 2010).

The key features of the adaptive immune response are specificity and memory (Male et al. 2007). Unlike the innate immunity response, the adaptive immune response is very specific and creates immunological memory after the first encounter with a specific pathogen, leading to an enhanced response to subsequent infections. The adaptive immune response is able to discriminate between extremely small differences in microorganisms or molecules. Lymphocytes B and T (B and T cells) and their products, mainly antibodies/immunoglobulins (Abs/Igs) (IgA, IgD, IgE, IgG and IgM) and some cytokines, are the main components of adaptive immunity and antigens are their targets (Abbas et al. 2015). B cells produce Abs, which recognize and deal extracellular pathogens and their products. However, T cells have a large number of activities: T helper (Th) cells regulate the development and production of Abs by B cells or interact with phagocytic cells, helping them to destroy the pathogens that they have phagocytosed, while cytotoxic T (Tc) cells recognize and destroy virus-infected and tumor cells.

Moreover, most immune responses consist of a wide variety of innate and adaptive components. In the early stages of infection, the innate response predominates but lymphocytes, acting in an integrated and coordinated form, subsequently begin to generate the adaptive response (Male et al. 2007).

2. Immune system of teleost fish

Teleost fish occupy a key position from the evolutionary point of view since they are the earliest class of vertebrates known to possess both innate and adaptive immunity response (based on the rag system) (Whyte 2007; Travis 2009). The immune system of teleost fish shows much similarity with that of higher vertebrates, besides small differences. Because fish are free-living organisms from the early embryonic stages of life and that they live in aquatic environments, they have developed a robust innate immune system for survival in the face of a variety of microorganisms (Rombout et al. 2005) mainly in low temperature conditions (fish are poikilothermic) since the adaptive immune response is dependent on temperature (Cuchens and Clem 1977; Avtalion and Clem 1981; Abruzzini et al. 1982; Clem et al. 1984; Miller and Clem 1984; Clem et al. 1985; Clem et al. 1991). Moreover, the immune response of wild and farmed fish can be influenced by a variety of parameters, among which temperature, stress management, fish density, light intensity, water quality, salinity, food or immunostimulants should be mentioned (Cuchens and Clem 1977; Avtalion and Clem 1981; Abruzzini et al. 1982; Clem et al. 1991; Magnadottir 2006; Van Muiswinkel and Vervoorn-Van Der Wal 2006; Magnadottir 2010; Hoseinifar et al. 2015).

The immune response is composed of physical and chemical barriers and cellular and humoral agents. In innate immunity, the epithelium and mucosal tissues represent the physical barriers, while phagocytes, non-specific cytotoxic cells and eosinophilic cells, including mast cells (MCs), represent the cellular effectors, and a variety of molecules (the complement, acute phase proteins and cytokines) direct the humoral immune response. On the other hand, adaptive immunity is directed by B and T cells as the cellular components, and Ig, IgM, IgD and IgT/Z as the humoral components, with IgM being the most common form found in teleost species (Rubio-Godoy 2010).

Side by side with the physical and chemical barriers, PRRs and the complement system are the very first line of protection of fish immunity. TLRs are considered the principal inducers of the innate immune response, while the complement stimulates the adaptive immune response. Moreover, the complement prevents the attachment, invasion or proliferation of pathogens (Bayne et al. 2001a; Ellis 2001; Whyte 2007). After PAMPs recognition by PRRs, initiate an intracellular signal transduction that ends with the expression of inflammatory genes, antiviral response and the maturation of dendritic cells (Whyte 2007).

The head kidney acts as a lymphohematopoietic organ and is the equivalent of bone marrow in higher vertebrates, which is lacking in teleost fish. Head kidney is the anterior or cephalic part of the kidney, in which the middle and posterior part are mainly tubular, with scarce immune cells (Zapata et al. 1997). In general, lymphohematopoietic cells are scattered at random throughout a stroma of fibroblastic reticular cells and sinusoidal blood vessels (Zapata et al. 1997). Among other leukocyte components, precursor cells, macrophages, neutrophils/acidophilic granulocytes (AGs) and lymphocytes are present in the head kidney as has been described in gilthead seabream (Sepulcre et al. 2002). In this specie, AGs are the major cell type participating in innate host responses, being the head kidney the principal immune organ that acts as the main source for this cell type (Sepulcre et al. 2002). As in most evolutionary advanced vertebrates, the lymphoid organs, thymus and spleen, are also present in teleost fish. The thymus suffers involution and produces T cells, while the absence of lymph nodes in fish has led it to be speculated that the spleen could be considered as an antigen-presenting organ that initiates the adaptive immune response (Chaves-Pozo et al. 2005b).

2.1. Innate immune system of teleost fish

The innate immune system of teleost fish is the first barrier of host against pathogens and it deals with any foreign material until the adaptive immune system is potent enough to take over (Fearon and Locksley 1996; Dixon and Stet 2001; Whyte 2007). The innate immune response is thought to be essential in combating diseases in fish due to the limitations of the adaptive immune response by their

poikilothermic nature and their limited antibody repertoire, affinity maturation and memory and relatively slow lymphocytes proliferation (Magnadottir 2006; Whyte 2007; Uribe et al. 2011).

When innate immune leukocytes sense the presence of pathogens or tissue damage, inflammation appears as the innate immune response to avoid pathogen replication or infection and to initiate the process of tissue repair. Inflammation provokes redness, heat (both due to blood flow and core temperature), swelling (accumulation of fluid), pain (release of chemicals) and loss of function (multiple causes) in the inflammatory site due to the high recruitment of leukocytes and the changes in the vascular components. Although each type of leukocyte has a different role, they work together coordinately.

As in mammals, innate immune system of teleost fish includes physical/chemical barriers, cellular and humoral components (Magnadottir 2006; Uribe et al. 2011).

2.1.1. Physical/chemical barriers

The skin and the presence of scales or the secretion of toxic substances, as well as the mucus, gills and the gastrointestinal tract represent defensive mechanisms. Moreover, there is a wide variety of humoral effectors that act in the innate of fish (Alexander and Ingram 1992).

2.1.2. Cellular components

The innate leukocyte populations of fish are constituted by i) non-specific cytotoxic cells (equivalent to mammalian NK lymphocytes), ii) monocytes/macrophages, iii) neutrophils/AGs, iv) MCs and v) others nuclear leukocytes.

Non-specific cytotoxic cells are agranular and small cells (Uribe et al. 2011). They have been found in peripheral blood and fluid, thymus, spleen and kidney (Rubio-Godoy 2010). They present cytotoxic activity against normal or transformed cell lines, virus-infected cells or parasitic protozoa (Utke et al. 2007). They release a variety of cytokines to eliminate the pathogens (Evans and Jaso-Friedmann 1992).

Monocytes/macrophages are large leukocytes that can present different body shapes, but are mainly round or horseshoe shaped. They are found in head kidney, connective tissue, spleen, peritoneal exudate, gills and thymus (Page and Rowley 1984; Sakai 1984; Bodammer 1986; Olivier et al. 1986; Suzuki 1986; Temkin and McMillan 1986; Olivier et al. 1992; Lieschke et al. 2001). In some teleost species dark coloured-macrophages form clusters in the lymphohematopoietic tissues; those clusters are called melanomacrophage centres (Agius 1980; Herraez and Zapata 1991). They phagocyte pathogens

and present oxidative burst in order to combat infection (Uribe et al. 2011) and to release a variety of cytokines (Ellis 1998). In gilthead seabream, macrophages have been found to migrate to the injury site, to phagocyte pathogens after cytokine stimulation, to produce reactive oxygen and nitric species (ROS/RNS) against PAMPs and to possess bactericidal activity (Mulero and Meseguer 1998).

Neutrophils/AGs have a round shape and a large content of granular vesicles. They are mainly found in the hematopoietic tissues and the peritoneal exudate, but also in blood, intestinal submucosa, skin, gill and gonads (Bullock 1963; Ezeasor and Stokoe 1980; Chaves-Pozo et al. 2003; Mulero et al. 2007b). Together with macrophages, neutrophils/AGs are responsible for phagocytosis in teleost fish (Sepulcre et al. 2002). They can also produce ROS as a mechanism of defense against pathogens. More specifically, studies in gilthead seabream have described AGs as the most active and abundant phagocytic cell type of this specie (Sepulcre et al. 2002), and are considered as functionally equivalent to mammalian neutrophils (Sepulcre et al. 2002). Moreover, AGs show strong phagocytic and ROS production capabilities (Sepulcre et al. 2002; Sepulcre et al. 2007a), produce cytokines in response to several immunological stimuli (Chaves-Pozo et al. 2004; Sepulcre et al. 2007a) and express a broad range of TLRs (Sepulcre et al. 2007a).

2.1.3.Humoral components

There are many humoral components participating in fish innate immunity. The most important regulatory molecules involved in this process include the complement system, lysozyme, lectins, growth inhibitors, antimicrobial polypeptides, natural antibodies, and protease inhibitors.

The *complement system* is responsible for three immune functions: i) opsonization, ii) inflammation by stimulating the contraction of smooth muscle, vasodilation and the chemoattraction of leukocytes, and iii) the lysis of pathogens through perforation of their membranes. The third component of the complement system (C3), which is mainly produced in liver (Boshra et al. 2006), is essential in the activation of this system (Rubio-Godoy 2010). After activation of C3, it is divided into C3a and C3b, the latter being the main phagocytosis-promoting factor. Macrophages and neutrophils have been found to possess specific receptors for the complement (Yano 1997). Like that of higher vertebrates, it can be activated by three pathways (the classic, alternative and lectin pathways) (Sakai 1992; Sunyer and Lambris 1998; Holland and Lambris 2002).

Lysozyme is a bacteriolytic enzyme widely distributed throughout the body (Uribe et al. 2011). It acts as a lytic enzyme of the peptidoglycan component of the bacterial cell wall and can also have an opsonic function. The sources of lysozyme are monocytes/macrophages, neutrophils and MCs of the intestine (Uribe et al. 2011).

Lectins are proteins or glycoproteins of non-immune origin that bind to particular carbohydrates, leading to their recognition. They participate in the recruitment of microorganisms and the precipitation of different substances (Arason 1996; Russell and Lumsden 2005), can activate the complement system (Rubio-Godoy 2010). They are classified into at least six families: legume lectins, cereal lectins, P-, S-, C-type lectins and antimicrobial polypeptides, such as histone H2B and three 60S ribosomal proteins, L40, L36A and L35, appear in integumental secretions of fish and in the skin mucus (Robinette et al. 1998; Cole et al. 2000; Patrzykat et al. 2001; Robinette and Noga 2001; Noga et al. 2002; Bergsson et al. 2005), and they participate in fish immunity against bacteria or viruses (Ingram 1980; Ellis 2001; Chinchar et al. 2004). Piscidins are antimicrobial polypeptides of 22 aminoacids forming an alpha helix with potent, broad-spectrum antibacterial activity against fish pathogens (Silphaduang and Noga 2001; Silphaduang et al. 2006). They are found in mucous tissues and immune cells (Rubio-Godoy 2010; Crivellato et al. 2015). Piscidins are inhibitors of the cell wall, nucleic acids, and protein synthesis or even enzymatic activity of bacteria (Campagna et al. 2007; Mulero et al. 2008a).

Transferrin and Interferon (IFN) act as *growth inhibitors*. Transferrin binds host's available iron inhibiting bacterial growth and also activates macrophages (Magnadottir 2006). IFN induces the expression of antiviral proteins (Ellis 2001; Magnadottir 2006).

Natural antibodies are produced at low regulated levels in the absence of antigenic stimulation of cells in healthy individuals (Whyte 2007; Uribe et al. 2011). They are found at high levels in the serum (Magnadottir et al. 1999a; Magnadottir et al. 1999b; Boes 2000; Ochsenbein and Zinkernagel 2000; Lange et al. 2001; Sinyakov et al. 2002), providing protection against bacterial, viral and fungal infections. In mammals, they are generated by B-1 cells (CD5+), a subset of long-lived, self-replenishing B cells active during embryonic and early developmental stages (Baumgarth et al. 2005). The intensity of production of natural antibodies depends on the species and the environmental conditions (Whyte 2007). Natural antibodies are key factors not only in innate immunity but also in adaptive immunity.

Protease inhibitors are found in the serum and other body fluids of fish (Bowden et al. 1997). Their function is to maintain fluid homeostasis and they are involved in acute phase reactions and against proteolytic secreting-pathogens (Magnadottir 2010).

2.1.4.Mechanisms of the innate immune system

The main mechanisms of the innate immunity developed in teleost fish to destroy harmful stimuli include: degranulation, chemotaxis, phagocytosis, oxidative burst, and opsonic and haemolityc serum activities.

Degranulation involves the release of a wide battery of immune regulatory mediators and antimicrobial cytotoxic molecules, from the granules present in MCs and neutrophils/AGs. These molecules subsequently induce *chemotaxis* or leukocyte extravasation to the site of infection in order to recruit other leukocytes and eliminate pathogens.

Phagocytosis is a biological process in which cells (phagocytes) engulfs solid particles or pathogens. In the process, recruited phagocytes come into contact with microbes at the inflamed tissue site. The PRRs present at the cell-surface recognize PAMPs and then form a PAMP-PRR complex. Subsequently, in a rearrangement of the actin-myosin cytoskeleton, the PAMP-PRR complex is endocytosed and directed to the lysosomes to produce a phagolysosome that kills the microbes inside the phagocytes. Phagocytosis in fish is mainly mediated by macrophages and neutrophils (Secombes and Fletcher 1992; Kemenade et al. 1994). Nevertheless, it has been reported phagocytic activity in fish dendritic cells (Esteban et al. 2015), B cells (Sunyer 2013) and thrombocytes (Meseguer et al. 2002).

The *oxidative burst* takes part in degranulation and chemotaxis processes since the release of ROS/RNS induce the recruitment of leukocytes, but also in phagocytosis since it helps in the elimination of the phagolysosomes. Some authors consider that this activity is only present in macrophages and neutrophils (Swindle et al. 2002); however, some studies have demonstrated that ROS/RNS are also produced by MCs (Brooks et al. 1999; Urb and Sheppard 2012). As in mammals, during the respiratory burst different ROSs are produced in fish, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), peroxinitrite ($ONOO^-$) and hypoclorous acid (OCl^-) (Figure 1).

ROS and RNS, such as superoxide anion, hydrogen peroxide and nitric oxide, are produced by neutrophils and macrophages, and have physiological, toxicological and immunoregulatory effects (Moncada et al. 1991; Lincoln et al. 1997) Superoxide ion and hydrogen peroxide are generated by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Swindle et al. 2004) and nitric oxide formation needs nitric oxide synthase (type II NOS) (Koranteng et al. 2000). In immune responses, when neutrophils and macrophages are activated, ROS/RNS are produced at relatively high levels (Koranteng et al. 2000).

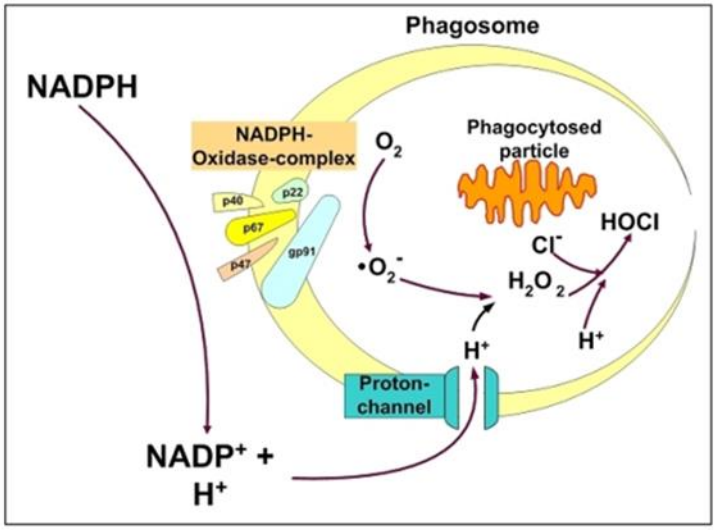

Figure 1. Reactive oxygen intermediates production during the respiratory burst (Riechelmann 2004).

Opsonization is the molecular mechanism whereby molecules, microbes or apoptotic cells are chemically modified to enhance their interaction with cell surface receptors on phagocytes or nonspecific cytotoxic cells. Moreover, it has been described that the complement is able to neutralize viruses by enveloping them (Whyte 2007). Opsonization also helps phagocytic efficacy through the plasma derived complement C3b (Sakai 1984; Matsuyama et al. 1992; Ellis 1999; Ellis 2001).

The complement is also responsible for the *haemolytic activity of serum* that produces the lysis of foreign cells and pathogens to avoid infection or pathogen replication (Whyte 2007). In fish, haemolytic activity is more active and heat labile than in mammals (Whyte 2007).

2.1.5.Receptors of the innate immune system

The innate immune system is the first line of host defense and is independent of a previous antigen contact. It is based on the recognition of different PAMPs (including polysaccharide, LPS, peptidoglycans, bacterial DNA or double stranded viral RNA) or DAMPs, presents in pathogenic organisms by germline-encoded PRRs or pattern recognition proteins (PRPs). PAMPs/DAMPs recognition and consequent PRR results in the activation of the transcription of inflammatory genes (Whyte 2007) and can undergo opsonization, phagocytosys, stimulation of cytotoxic cells, activation of complement cascades and activation of lytic pathways (Magnadottir 2006).

PRRs include: i) soluble components, such as the complement protein C3, lectins, the C-reactive protein and the serum amyloid protein (Magnadottir 2006; Magnadottir 2010); ii) cytoplasmic receptors, such as retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NRLs) (Kumar et al. 2009; Takeuchi and Akira 2010)

and iii) transmembrane receptors of immune cells, such as TLRs which recognize PAMPs in different cellular compartments (Kumar et al. 2009; Takeuchi and Akira 2010). In teleost, it has been reported the presence of TLRs homologs to their mammalian counterpart ones, since these receptors are highly conserved (Roach et al. 2005), as well as the presence of TLRs only present in lower evolutionary advanced organisms like i.e.: soluble TLR5, TLR21 and TLR 22 (Roach et al. 2005). In particular, it has been reported the presence of TLRs in gilthead seabream AGs and macrophages (Sepulcre et al. 2007a).

2.2. Adaptive immune system of teleost

The appearance of functional lymphocytes let teleost fish develop a specific capacity to recognize particular antigenic insults, become activated in their presence, and show memory immune responses (Rubio-Godoy 2010). Together, lymphocytes analogous to the mammalian B and T cells, and the humoral components, Ig and cytokines, of the adaptive immunity allow a rapid and an efficient pathogen elimination upon secondary exposure to the same antigen (Rubio-Godoy 2010).

2.2.1. Cellular components

The immunological memory of B and T cells allows teleost fish to present a more competitive response against successive exposures against pathogens. This feature of the adaptive immune response confers protection to fish from pathogens through vaccines (Uribe et al. 2011).

B cells are mainly produced in the head kidney, followed by the thymus and spleen of teleost fish (Koumans-van Diepen et al. 1995; dos Santos et al. 2000; Trede et al. 2001; Crowhurst et al. 2002; Salinas et al. 2011) and they populate peripheral organs such as the intestine (Rombout et al. 2005). There are some evidences showing that B cells are also developed in the pancreas (Danilova and Steiner 2002). Teleost B cells produce Igs to label specifically altered-host or foreign cells in order to agglutinate or precipitate soluble antigens, promoting phagocytosis (Rubio-Godoy 2010).

T cells are mainly produced in the thymus, followed by the head kidney and the spleen of teleost fish (dos Santos et al. 2000; Rombout et al. 2005; Salinas et al. 2011; Nakanishi et al. 2015) but they may also be found in skin, gills, gut or blood (Rombout et al. 2005). In mammals, T cells are categorized into two general populations: Tc and Th cells. The existence of Tc cells in fish, where they would be involved in specific cell-mediated cytotoxicity, similar to that which occurs in higher vertebrates has been suggested (Fischer et al. 2006). Recently, fish Tc and Th cells have been identified as CD8+ and CD4+ cells, respectively, in ginbuna crucian carp (*Carassius auratus langsdorfii*) (Toda et al. 2009; Toda et al. 2011). There is evidence of the existence of Th cells in teleost fish, similar to their mammalian counterparts (Secombes 2008; Yamaguchi et al. 2015), however, unlike mammalian

species, their polarization into Th1, Th2, Th17 and regulatory T cells has not been demonstrated yet. Whatever the case, teleost T cells would be involved in allograft rejection (ability of T cells to recognize genetically different major histocompatibility complex molecules of transplants and induce an inflammatory reaction), stimulation of phagocytosis and antibody production by B cells (Zapata and Amemiya 2000). As in mammals, the adaptive immune response of teleost T cells is thought to be mediated by the production of cytokines (Rubio-Godoy 2010; Uribe et al. 2011).

2.2.2.Humoral components

The way by which B cells defend the host body against pathogens involves the production of specific antibodies. Ig production by B cells results in protective responses against antigens through the neutralization of small molecular weight molecules by agglutination and/or precipitation of soluble antigens by the antibodies, working as opsonins, by coating antigens and promoting phagocytosis. Moreover, antibodies can activate the complement system when they bind to the antigens, producing conformational changes in the Fc region (Uribe et al. 2011). Furthermore, as mentioned in the innate immunity section, although fish have not been previously exposed to an exogenous specific antigen, B cells can produce low levels of Ig against this specific antigen, working as natural antibodies and helping the innate humoral immune system.

Teleost antibodies are found systemically in plasma (Uribe et al. 2011), skin (Cain et al. 2000; Hatten et al. 2001), intestine (Rombout et al. 1986; Jones et al. 1999), gill (Lumsden et al. 1993), and bile (Jenkins et al. 1994).

Three different Igs have been identified in fish: IgM (Acton et al. 1971), IgD (Wilson et al. 1997) and IgT/Z (Danilova et al. 2005; Hansen et al. 2005). A tetramer of the IgM class containing eight antigenic combining sites is the most predominant Ig in teleost fish (Acton et al. 1971). Also, a monomer of IgM has been found in the serum of some teleost fish (Wilson and Warr 1992). The IgM concentration in serum varies among teleost species (Vilain et al. 1984; Scapigliati et al. 1997; Uchida et al. 2000) and depends on the animal size (Sanchez et al. 1993; Magnadottir et al. 1999b), water temperature (Sanchez et al. 1993), water quality (Olesen and Jørgensen 1985) and season of the year (Olesen and Jørgensen 1985; Magnadottir et al. 2001). IgD was the second isotype identified in channel catfish (*Ictalurus punctatus*). As in mammals, fish IgD gene sequence is located immediately under the IgM gene and is expressed in B cells (Wilson et al. 1997). IgT was described in teleost fish (Danilova et al. 2005; Hansen et al. 2005), and is also named IgZ in cyprinids (Parra et al. 2016). B cells exclusively expressing surface IgT or IgM have been described in rainbow trout (Zhang et al. 2010) and B cells expressing IgZ or IgM in zebrafish (Schorpp et al. 2006; Page et al. 2013).

There is no evidence concerning the presence of IgE, IgA or IgG in teleost fish, although a functional high-affinity receptor for IgE has been described (Da'as et al. 2011), which, in mammals, acts as an IgE receptor. Moreover, although there are some evidences showing that antibodies found in mucus are slightly different structurally from serum Ig (Lobb and Clem 1981), a distinct class of secretory Ig analogous to mammalian IgA has not been found in fish.

T cells produce a variety of soluble factors, cytokines, to mediate cell interaction in order to defend the organism against pathogens. Although the existence of Th1 and Th2 cells in teleost fish has not yet been demonstrated, some evidence suggests that they could exist and have a similar role to their mammalian counterparts (Secombes 2008; Yamaguchi et al. 2015). Indeed, the cytokines, interferon-γ (IFN-γ) and interleukin-4 (IL-4), known to be produced by mammals Th1 and Th2 cells, respectively, seems to mediate similar Th1 and Th2 cells responses in teleost fish (Zou et al. 2005; Li et al. 2007; Rubio-Godoy 2010).

2.3. Effect of environmental elements on fish immune system

Several endogenous and exogenous factors can affect to the immune system. External factors including environmental temperature (Nikoskelainen et al. 2004), pollution (Köllner et al. 2002), handling (Montero et al. 1999; Ortuno et al. 2001), seasonal factors (Zapata et al. 1992), diets (Ortuño et al. 2001) and immunostimulants which activate the PRR pathway (Mulero et al. 1998; Cuesta et al. 2003) affect to the fish immunity, in particular, to the innate immune system. Fish immune response is highly influenced by temperature due to its poikilothermic nature and since the adaptive immune response is dependent on temperature, (Le Morvan et al. 1998).

Among the inherent factors, age, sex, maternal transfer, genetic variation and evolutionary linage can affect to disease resistance and immune system of fish. It has been observed an increasing plasma IgM concentration with age, as well as, different IgM levels (Magnadottir 2010) and phagocytic and complement activities depending on sex hormones (Cuesta et al. 2007). Related to maternal transfer, it has been studied different immune parameters that are maternally transferred during the offspring and seems to be involved in the immune defense of embryos and larvae (Mulero et al. 2007a).

3. Regulatory molecules of the immune

3.1. Cytokines

Cytokines are a large group of glycoproteins that exert different functions within both the innate and adaptive immunity, such as, i) activation of effectors cells (phagocytes and lymphocytes) and ii) growth, differentiation and attraction of immune cells (Abbas et al. 2015). These glycoproteins with low molecular weight (8-25KDa) are secreted in response to different stimuli by white blood cells,

principally, by Th cells and macrophages. Cytokines show some characteristics: i) may exert autocrine, paracrine and endocrine action by binding to a specific receptor whom elicits a signal that results in the modulation of the expression of different genes, including genes involved in the secretion of other cytokines; ii) a particular cytokine can modify the activity of several cells; iii) different cells types can synthesize the same cytokine; iv) pleiotropy action, a cytokine has different biological effect on cells; v) different cytokines with the same action; vi) synergistic effect, when different cytokines together exert a biggest action than the individual one; vii) antagonist effect, when two cytokines present opposite actions; viii) cytokine secretion is short-live; ix) low constitutive production; x) exert its action on the target cell through binding to high affinity specific receptors and xi) a specific cytokine can stimulate or inhibit the expression of other cytokine (Secombes et al. 1996; Kindt et al. 2007).

Cytokines are classified into four structural families, including, the hematopoietin or interleukin (IL) family, the interferon (IFN) family, the tumor necrosis factor (TNF) family and the chemokine family (Kindt et al. 2007; Male et al. 2007).

Interleukin are classified into six families (1, 2, 6, 10, 12 and 17) (Savan and Sakai 2006). Those of the IL-1 family participate in pro-inflammatory processes (Dinarello 1997; Dinarello 1998a; Dinarello 1998b) and include IL-1β and IL-18 (Savan and Sakai 2006). IL-1β, which are constitutively expressed in macrophages and neutrophils/AGs in some teleost species (Engelsma et al. 2001; Fast et al. 2006; Fast et al. 2007), elicit the humoral response (Savan and Sakai 2006), while IL-18 is stored intracellularly in a vast range of cells, such as macrophages, dendritic cells, and B and T cells and induce interferon (IFN)-γ secretion (Whyte 2007). In the IL-2 family, IL-2 has been found to modulate the production of IFN-γ (Diaz-Rosales et al. 2009) and drive lymphocyte differentiation (Corripio-Miyar et al. 2012). IL-12 is also an important inflammatory mediator as it induces IFN-γ secretion (Whyte 2007). IL-6, a member of the IL-6 family (Savan and Sakai 2006), is involved in inflammatory processes (Zante et al. 2015) and IL-10, from the IL-10 family (Savan and Sakai 2006), acts as an anti-inflammatory cytokine, inhibiting the production of pro-inflammatory innate immune mediators, such as IL-1 (Karan et al. 2016). In the IL-17 family, IL-17 has been found to regulate the production of IL-6 and IL-8 (Monte et al. 2013) and to be regulated by, the still not fully characterized, regulatory T cells (Secombes et al. 2011).

TNF-α is an important macrophage-activation factor produced by leukocytes that lead to increased respiratory activity, phagocytosis and RNS(Whyte 2007; Uribe et al. 2011). TNF-α also induce the expression of various immune genes including IL-1β, IL-8 and cyclooxygenase 2 (Zou et al. 2003).

IFN-α and *IFN-β* play and essential role in the defense against viral infection by inhibiting the nuclear acid replication of viruses within infected cells (Robertsen 2006; Uribe et al. 2011) or protecting

other cells from viral infection by modulating the expression of a variety of genes, some of them encoding for antiviral proteins (De Veer et al. 2001; Samuel 2001). IFN-γ is produced by non-specific cytotoxic cells and T cells (Rubio-Godoy 2010) in response to IL-12, IL-18, mitogens or antigens (Whyte 2007), and has the same function as its mammalian homologue (Zou and Secombes 2011).

Chemokines are a superfamily of cytokines produced by different cell types. They possess chemoattractant properties that induce the recruitment, activation and adhesion of cells to sites of infection or tissue damage, and are crucial in changing the innate immune response into the adaptive response (Whyte 2007). This family is divided into CXC, CC, C and CX3C classes. IL-8, the first fish chemokine known, induces the recruitment of neutrophils, T cells and basophils, increases cytosolic calcium levels and respiratory burst, and produces changes in neutrophil shape (Whyte 2007).

3.2. Eicosanoids

Eicosanoids, are lipids mediators with a key role in the immunity response and exert their action as a mediators and second messengers in different biological process such as, haemostasis, inflammation, mobilization of leukocytes, phagocytosis and regulation of the immune system (Rubio-Godoy 2010). Eicosanoids including prostaglandins (PGs), thromboxanes (TXs), prostacyclins (PCs), leukotrienes (LT), lipoxins (LPs) and endocannabinoids, are produced by derivates of myeloid lineage (Harizi and Gualde 2004) and non-immune cells.

Prostanoids are synthesized from AA. Different stimuli, such as, bacterial peptide or cytokines, trigger the liberation of AA by PLA_2 from the cell membrane. Cyclooxygenase enzymes (COX), converts free AA into PGH_2. Then PGH_2 is converted to PGs, TXs and PCs by different enzymes or by spontaneous reaction (Figure 2). PGs have been implicated in all stages of inflammation, eliciting a pro- or anti-inflammatory response (Lone and Taskén 2013). These lipids mediators modulate effector and putative accessory cell function in fish (see section 4) (Secombes et al. 2001a).

Leukotriens and lipoxins are synthetisazed from AA by lipoxygenase enzyme (5-LO and 15-LO, respectively) resulting in the production of 5-hydroxyperoxy-eicosatetraenoic acid (5-HPETE) or 15-HPETE intermediates. These intermediates are the precursors of LTs and LPs, with pro-inflammatory and anti-inflammatory activities, respectively, (Figure 2) (Cabral 2005).

Endocannabinoids acts as an agonist of inhibitory GPR (iGPR). Little is known about the role of this lipids mediators on the innmune systems (Cabral 2005).

In fish, together with the AA, the eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are principal components of the cellular membrane (Rowley et al. 1995).

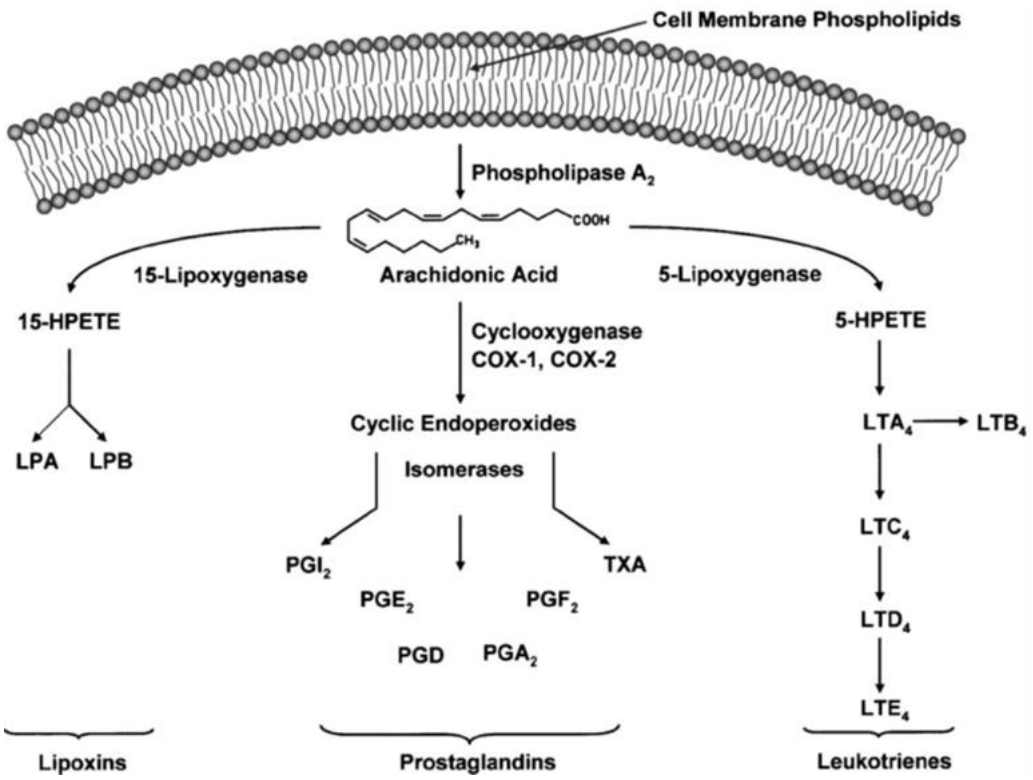

Figure 2. Biosynthetic pathway of eicosanoids (Cabral 2005)

3.2.1.Prostaglandin receptors

All PGs, with the exception of some cyclopentenote PGs (CyPGs), signal through the interaction with rodopsin-like seven transmembrane spanning G protein-coupled cell surface receptors (GPRs) subfamily and constitute the prostanoid receptor subfamily (Hata and Breyer 2004). CyPGs are transported into the cell and modulate gene expression since they have no cell surface receptors (Negishi and Katoh 2002). Prostanoid receptor subfamily is composed by nine members (DP1, CRTH2 or DP2, EP1-4, FP, IP and TP) (Breyer and Breyer 2001; Breyer et al. 2001). Different prostanoids can signal by different prostanoid receptors, since these subfamily receptors share specific common motifs. Moreover, one prostaglandin can act as pro-inflammatory and anti-inflammatory mediator depending on multiple factors which determine the specific role of the prostaglandin receptor (Hata and Breyer 2004).

The nine members of the prostanoid receptor subfamily are divided into 3 group or subfamilies according to the sequence identity and the signaling pathways that trigger (Toh et al. 1995). Although there is a 20-30% of sequence identity between each prostanoid receptor (Hata and Breyer 2004; Hirata and Narumiya 2011a), it has been demonstrated that receptors that trigger common signal pathway show higher sequence identity (Figure 3) (Jabbour and Sales 2004; Hirata and Narumiya 2012b). The subfamily of relaxant receptors includes the IP, DP, EP2 and EP4 receptors (Figure 3) which produce

the relaxation of the smooth muscle. Those receptors are coupled to a Gαs-type G protein leading to adenylyl cyclase (AC) stimulation and the consequent production of intracellular cyclic adenosine monophosphate (cAMP) (Figure 4) (Hata and Breyer 2004; Jabbour and Sales 2004; Hirata and Narumiya 2011a; Hirata and Narumiya 2012b). The subfamily of contractile receptors includes the TP, FP and EP1 receptors (Figure 3) which provoke the contraction of the smooth muscle and they are coupled to a Gαq-type G protein triggering cytosolic Ca^{2+} mobilization (Figure 4) (Hata and Breyer 2004; Jabbour and Sales 2004; Hirata and Narumiya 2011a; Hirata and Narumiya 2012b). EP3 receptor also known as inhibitory receptor, can cause both relaxation (by increasing cAMP levels) and contraction (by decreasing cAMP level and Ca^{2+} mobilization) of the smooth muscle depending on the splice variant activated and the cell type (Figure 3 and 4) (Hata and Breyer 2004; Jabbour and Sales 2004; Hirata and Narumiya 2011a). However, this later group signal mainly by coupling with Gi-type G proteins and eliciting the AC inhibition and reduction of cAMP level (Hirata and Narumiya 2012b). DP2 receptor mediates the inhibition of cAMP level and Ca^{2+} mobilization coupled to a Gi-type G protein (Figure 3 and 4) (Hirai et al. 2001a; Sawyer et al. 2002; Hirata and Narumiya 2011a).

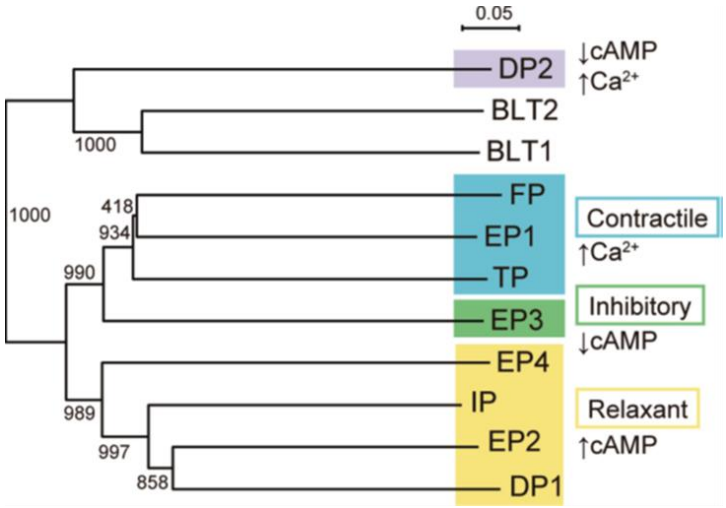

Figure 3. Phylogenetic tree of prostanoids receptors subfamilies in human. Prostanoids receptors subfamily is divided into 3 groups according to the sequence identity and the signaling pathways (Hirata and Narumiya 2011a).

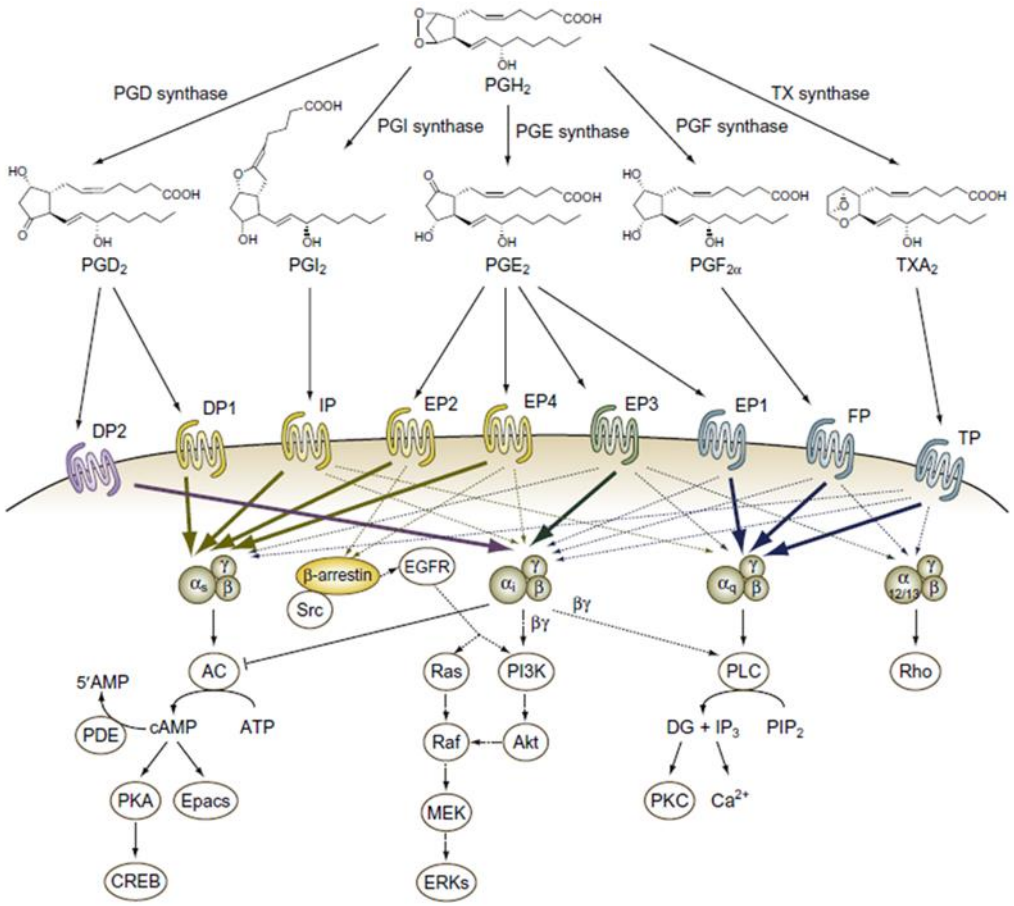

Figure 4. Prostanoid synthesis pathway and the signaling pathway activated depending on the predominant prostanoid receptors coupled (bold arrows) (Hirata and Narumiya 2012b).

PGD_2 binds to DP1 and CRTH2 or DP2 receptors with almost the same affinity (Nagata et al. 1999; Hirai et al. 2001a) eliciting different effects according to the activated receptor. While DP1 is a prostanoid receptor subfamily member sharing sequence identity with IP and EP2 receptors (relaxant prostanoid receptors); DP2, initially described as a Th2 cell-specific surface receptor (CRTH2) shares high similarity with other chemoattractant receptors and shows very low similarity with the DP receptors. DP1 receptor show high affinity to PGD_2 and to PGJ_2. DP2 receptors show high affinity to PGD_2 and $15dPGJ_2$, (Hirai et al. 2001a; Sawyer et al. 2002).

PGE_2 exerts different biological effects depending on the EP receptor coupled and for instance, the signal transduction pathways activated (Harris et al. 2002b). EP receptors are encoded by four different genes (Narumiya et al. 1999). EP1-EP4 receptors binds PGE_2 with different affinities, EP3 and EP4 receptors show highest affinities than EP1 and EP2 (Abramovitz et al. 2000). Several C-terminal splice variants of EP3 receptor has been described which lead different signal transduction pathways (Breyer and Breyer 2001; Breyer et al. 2001).

CyPGs, $15dPGJ_2$ and $\Delta 12\text{-}PGJ_2$, can enters into the cell by a transport system (Harris et al. 2002b; Negishi and Katoh 2002) or can be synthesized from PGD_2 (Harris et al. 2002b). $15dPGJ_2$ exerts its action through PPARγ (a member of the nuclear receptor superfamily) which is involved in lipid metabolism and inflammation (Harris et al. 2002b; Negishi and Katoh 2002). Activated PPARγ binds to retinoid X receptor (RXR) to form a heterodimer. This heterodimer is translocated into the cellular nucleus where recognizes and binds to a peroxisome proliferator responsive elements (PPREs) located in the promoter region of target genes (Kliewer and Willson 1998). $15dPGJ_2$ can also interact with DP2 leading the inhibition of cAMP level and Ca^{2+} mobilization (Hirata and Narumiya 2012b). In contrast, PPARγ can be inhibited by phosporilation mediated by $PGF_2\alpha$ (Negishi and Katoh 2002). Moreover, this PG possess two β carbons in its structure which confers the electrophilicity and can be attack by thiols groups of different proteins and nucleics acids. Due to the electrophilic structure of $15dPGJ_2$, it can form covalent Michael adducts with proteins and modify its structure and biological function, giving rise to the receptor independent signaling pathway (Pérez-Sala 2011).

3.2.2. Role of prostaglandins in mammals' immunity and immunopathology

Prostaglandins have key roles and, in some cases opposing effects, in several immunopathologycal and in physiological process being cells of the innate immune system the main source of PGs (Hirata and Narumiya 2012b). The effects of PGs depend on the microenvironment, cell type, receptor expression profile and ligand affinity (Park and Christman 2006).

PGE_2 has been classically consider an anti-inflammatory mediator, but actually, it is known that this PG has a pleiotropic effect, acting as a pro or anti-inflammatory mediator. In macrophages, the activation of EP2 and EP4 receptors by PGE_2 inhibit TNFα production whereas, mRNA levels of IL10 is up-regulated, showing stimulating effects for type-2 immune responses (Harris et al. 2002b; Harizi et al. 2003). In neutrophils, this PG has an anti-inflammatory effect (Wright et al. 2010). It is known that mitogenesis of T-cells is suppressed by PGE_2, while apoptosis is induced or inhibited by this PG in mature resting or in activated T cells respectively (Harris et al. 2002b). Similarly, PGE_2 suppresses immature B-cells proliferation, while mature B cells proliferation and immature B cells apoptosis are activated (Harris et al. 2002b). PGE_2 induces the migration of colorectal cancer cells (Buchanan et al. 2006), 2006, lung cancer cells (Kim et al. 2010) and maturation of skin Langerhans cells (Kabashima et al. 2003) signaling via EP4 receptor. This PG is also involved in several immunopathology, such as, rheumatoid arthritis and allergic asthma. High concentration of PGE_2 has been found in rheumatoid arthritis synovial fluids, whereas, in allergic asthma, PGE_2 has a bronchodilatory effect and inhibits the release of histamine, showing a protective role in this context (Park and Christman 2006).

PGD_2 is the most frequent PG in the central nervous system and peripheral tissues and regulates physiological and inflammatory responses. It has been reported an anti-inflammatory effect of this PG. PGD_2 inhibit dendritic cells and skin Langerhans cell migration mediated by interaction through DP1 receptor (Hammad et al. 2007; Ricciotti and FitzGerald 2011). On the other hand, it has been described a pro-inflammatory effects of this PG. It has been found high concentration of PGD_2 in allergic inflammation sites being mast cells and activated macrophages the main source of this PG (Park and Christman 2006). In allergic inflammation, when PGD_2 binds to DP1 triggers vasodilatation, edema, bronchoconstriction leading nasal congestion (Morteau 2000; Park and Christman 2006; Hirata and Narumiya 2012b). Intradermal administration of PGD_2 can cause erythema (Sandig et al. 2007).

CyPG has been classically defined as an anti-inflammatory mediator. Animals treated with COX inhibitor can trigger the resolution of the inflammation after administration $15dPGJ_2$ (Sandig et al. 2007). It was reported that this PG has a pro-apoptotic and anti-proliferative effects. In lung, breast or colon cancer cells it has been described the pro-apoptotic effects of CyPG (Garzon et al. 2011), whereas, some mechanisms, such as the induction of oxidative stress mediates the anti-proliferative activity of this PG (Garzon et al. 2011). In addition, CyPGs exert anti-viral effects (Santoro 1997). However, some studies points to the implication of $15dPGJ_2$ in the progress of human arthritis (Shan et al. 2004) and Parkinson's disease (Pierre et al. 2009).

4. The role of prostaglandins in the regulation of fish immunity

4.1. Prostaglandin biosynthetic pathway in fish

Prostaglandins (PGs) play a significant role in the progress of the immune response at multiple levels, often in concert with microbial ligands, cytokines and various other factors (Simmons et al. 2004b). They are small fatty acid molecules derived from polyunsaturated fatty acids released from the cell membrane phospholipids through the action of the enzyme PLA_2 (Figure 5). Depending on the fatty acid precursor, different series of PGs will be produced: series 2 PGs are produced from AA and series 3 PGs are produced from EPA. So the first limiting step in PG biosynthesis is the bioavailability of these fatty acids. The second limiting step in PG biosynthesis concerns the enzymes cyclooxygenases (COX, also known as prostaglandin G/H synthase and prostaglandin-endoperoxide synthase 2) which convert AA or EPA to PGH_2 or PGH_3, respectively (Figure 5). At least two COX isoforms exist, the constitutive (COX-1) and inducible (COX-2) isoforms. COX-1 is expressed in many cell types distributed throughout the body, whereas COX-2 expression is highly restricted under basal conditions and upregulated during inflammation in different cell types (Simmons et al. 2004b). The first study demonstrating the presence of the existence of one inducible form of COX in fish was reported by Zou and coworkers in 1999 in goldfish (Zou et al. 1999). To date, many studies have demonstrated the presence of mammalian homologs for COX-2 in a variety of fish species and their modulation under stimulation conditions; for example in zebrafish (*Danio rerio*) (Grosser et al. 2002a), trout

(*Oncorhynchus mykiss*) (Boltaña et al. 2011; Belmonte et al. 2014b), seabream (*Sparus aurata*, L.) (Sepulcre et al. 2007a; Mulero et al. 2008c; Sepulcre et al. 2011b; López-Muñoz et al. 2012; Boltana et al. 2014b), sea bass (*Dicentrarchus labrax*) (Sepulcre et al. 2007c), Atlantic cod (*Gadus orhua*) (Holen et al. 2012a) and Atlantic salmon (*Salmo salar*) (Fast et al. 2005a). However, few studies have been focused on the correlation between COX-2 expression and PG production.

PGH_2 is converted into PGE_2 by the action of PGE synthase (PGES, Figure 5) (Joo and Sadikot 2012b). In mammals, there are several isoforms of this enzyme: the cytosolic PGES (cPGES) and the 6 membrane-associated PGES or microsomal PGES (mPGES) (Murakami et al. 2002b). One of these synthases (mPGES-1) is a highly inducible and displays functional coupling with upstream COX-2 enzymes in cells (Murakami et al. 2002b). In zebrafish, the existence of mPGES-1 and c-PGES homologs has been reported along with an apparent correlation between mPGES-1 and COX-2 expression profile (Pini et al. 2005a). However, no reports exist about the contribution of this enzyme to PGE_2 production in zebrafish or other fish species.

PGH_2 is further converted, leading to the generation of PGD_2 through the action of PGD synthase (PGDS, Figure 5). In mammals, two distinct types of PGDS have been reported: the hematopoietic form (H-PGDS) and the lipophilic ligand-carrier protein (lipocalin)-type enzyme (L-PGDS). These two enzymes share no homology in DNA or polypeptide sequences (Kanaoka et al. 1997a), but both have been reported to play an important role in the immune response: H-PGDS participate in the onset and resolution of acute inflammation (Rajakariar et al. 2007b), while L-PGDS is only detected in macrophages in a pathological environment (Cipollone et al. 2004). Previous studies showed that non-mammalian (zebrafish, xenopus and chicken) and mammalian L-PGDS formed an "L-PGDS sub-family" in the lipocalin gene family, while several orthologs for the mammalian L-PGDS exist in zebrafish (Fujimori et al. 2006). However, relatively little is known about PGD synthases in fish immunity. For example, Bayne et al. identified the presence of PGD synthase in livers of rainbow trout from fish challenged with a *Vibrio anguillarum* bacterin (Bayne et al. 2001a). Boltaña and coworkers showed the upregulation of the same gene in trout macrophages challenged with peptidoglycan and the production of PGD_2 by these cells but with different kinetics (Boltaña et al. 2011). Although not mentioned by the authors, this PGDS is homolog to mammalian L-PGDS. Several homologs for mammalian L-PGDS are available on the databases of the zebrafish genome. The study of the enzymatic activity of one of these homologs showed that it was not able to convert PGH_2 into PGD_2 (Fujimori et al. 2006). However, additional work is needed to clarify whether L-PGDS enzymes are responsible for PGD_2 synthesis in fish.

Finally, in the PG biosynthesis pathway, PGD_2 is converted to cyclopentenone derivates CcyPGs), e.g. $15dPGJ_2$ and $\Delta^{12}PGJ_2$, by albumin-independent and -dependent reactions, respectively

(Figure 5) (Shibata et al. 2002b). However, data on the production and the biological activity of these compounds in the fish immune response are scant.

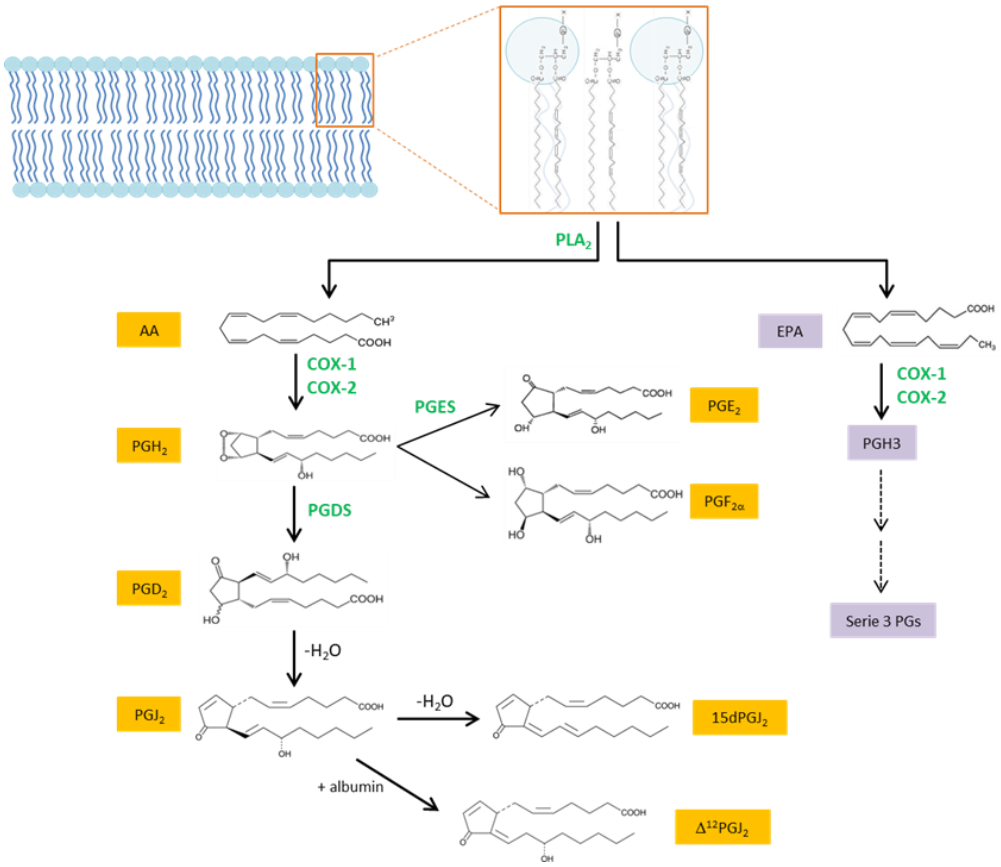

Figure 5. Prostaglandin biosynthetic pathway. Arachidonic acid (AA) or eicosapentaenoic acid (EPA) are first liberated from membrane glycerophospholipids by the actions of the phospholipase A_2 (PLA_2) family of enzymes. The cyclooxygenase (COX-1 and COX-2) enzymes then catalyze the biosynthesis of PGH_2 or PGH_3. PGH_2 and PGH_3 are subsequently converted to one of several structurally related prostaglandins of the serie 2 and serie 3 respectively, including PGE_2 or PGE_3, PGD_2, $PGF_{2\alpha}$, by the activity of specific PG synthases. PGD_2 is converted to cyclopentenone derivates: 15-deoxy-$\Delta^{12,14}$ PGJ_2 ($15dPGJ_2$) and Δ^{12} PGJ_2, by albumin-independent and -dependent reactions, respectively.

4.2.Prostaglandin production by fish immune cells

4.2.1. The in vitro approach

4.2.1.1. Arachidonic acid and eicosapentanoic acid in PG production by fish immune cells

The prostaglandin-generating ability of fish immune cells has received special attention especially in the 1990s. The first limiting step in PG biosynthesis is the bioavailability of cell-membrane derived fatty acids. Even today few reports exist concerning the availability of these fatty acids and their contribution to PG production in fish (Table 1). The earliest study in this respect was carried out by

Matsumoto and coworkers in 1989 using thrombocyte suspensions from different fish species: flounder (*Paralichthys olivaceus*), black seabream (*Acanthopagrus schlegeli*), black rockfish (*Sebastes schlegeli*), and red seabream (*Pagrus major*) (Matsumoto et al. 1989). Their study revealed that the incubation of thrombocytes with AA resulted in the production of $PGF_2\alpha$, PGE_2, and PGD_2 in all species studied, although only thrombocytes from black rockfish were able to metabolize the EPA, producing mainly PGE_3 and PGD_3. More recent reports have demonstrated that the exogenous administration of AA or/and EPA to fish leukocytes in culture significantly increases the PGE production in a concentration-dependent way. For example, in head kidney leukocytes from Atlantic cod, supplementation of cultures with a combination of AA and EPA promoted an increase in PGE_2 and PGE_3 production while, interestingly, supplementation with AA alone, only increased the levels of PGE_2 (Furne et al. 2013). Similar results were obtained using head kidney macrophages from yellow croaker (Li et al. 2012; Li et al. 2013) in which supplementation with AA enhanced the production of PGE_2 in a dose-dependent manner (Li et al. 2012), while only the highest doses of EPA had any effect on the generation of PGE_2 (Li et al. 2013). In the supplementation assays, AA and EPA are, presumably, incorporated into the cellular membranes as occurs with dietary fatty acids. Interestingly, the production of the soluble form of PLA_2 induced by AA, did not show the same kinetics as PGE_2 production (Li et al. 2012) and even decreased with EPA (Li et al. 2013), suggesting that this enzyme is not the only rate-limiting factor in the production of PGE_2 in these assays. Regarding the possible effect of PGE_2 on the inflammatory milieu, supplementation of head kidney macrophage cultures with AA or EPA also had no significant effect on the production of IL-1β, despite the increase in PGE_2 production (Li et al. 2012; Li et al. 2013). The lack of immune stimuli priming this process may account for this result (Weldon et al. 2007; Li et al. 2012; Li et al. 2013).

PG	Fish	Sample	Experimental conditions	Determination method	References
The *in vitro* approach					
$PGF_{2\alpha}$, PGE_2, PGD_2, PGE_3, PGD_3	Flounder, black seabream, black rockfish, and red seabream	Thrombocyte suspensions	AA or EPA	TLRCS and HPLC	(Matsumoto et al. 1989)
PGE_2 PGE_3	Atlantic cod	HK leukocytes	AA:EPA	LC-MS/MS	(Furne et al. 2013)
PGE_2,	Yellow croaker	HK macrophages	AA	EIA	(Li et al. 2012)
PGE_2,	Yellow croaker	HK macrophages	Incubated with EPA or DHA	EIA	(Li et al. 2013)
PGE_2, $PGF_{2\alpha}$, PGD_2	Trout	Macrophages and total HK leucocytes	Calcium ionophore and AA	HPLC GC-ECMS	(Rowley et al. 2012)
PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$	Trout	Blood cells suspensions	Calcium ionophore	GC-MS	(Pettitt et al. 1991)
PGE_2, PGE_3	Trout	Macrophages and total HK leucocytes	Calcium ionophore	EIA	(Secombes et al. 1994a)
PGE	Trout	Brain, heart, spleen, liver, gills, skin, eye, ovary, muscle suspension cells	Calcium ionophore	EIA	(Knight et al. 1995)
PGE	Trout	HK macrophages	Zymosan or calcium ionophore	ICGCECMA	(Knight et al. 1993b)
PGE	Trout	HK macrophages	Opsonized zymosan or complement fragments	EIA	(Rowley et al. 2012)
PGE_2	Turbot	Pronephric leukocytes	β-glucans	EIA	(Paramá et al. 2007)
PGE_2	Trout	HK macrophages	LPS combined or not with calcium ionophore	EIA	(Rowley et al. 1995)
PGE_2	Goldfish	HK macrophages and GMCL	LPS and/or MAF before challenge with calcium ionophore	EIA	(Zou et al. 1999)
PGE_2	Gilthead seabream	HK differentiated macrophages	AsLPS, VaLPS or EcLPS	EIA	(Boltana et al. 2014b)
PGE_2, PGE_3	Altlantic cod	HK leukocytes	LPS or Poly I:C	LCITMS	(Holen et al. 2012a)
PGE_2, PGD_2	Trout	Head kidney macrophages	PGN	EIA	(Boltaña et al. 2011)
The *in vivo* approach					
PGE_2	Trout	Peritoneal exudates	Injection or not of zymosan	EIA	(Rowley 1996)
PGE_2	Trout	Biopsies of kidney	Infection or not with proliferative kidney disease	EIA	(Rowley 1996)
PGE_2	Turbot	Ascitic fluid	Infection with *P.dicentrarchi*	EIA	(Paramá et al. 2007)
PGE_2	Gilthead seabream	Plasma	Injected with AsLPS or VaLPS	EIA	(Boltana et al. 2014b)

Table 1. Production of prostaglandins by fish immune cells

4.2.1.2. Role of calcium in PG production by fish immune cells

Another set of studies focused on the generation of PGs by fish leukocytes following challenge with calcium ionophores (Table 1). Most of these studies were performed by using rainbow trout cells, mainly head kidney macrophages (Knight et al. 1993b; Secombes et al. 1994a; Rowley et al. 1995; Rowley et al. 2012), and total head kidney leukocytes (Secombes et al. 1994a; Rowley et al. 2012), but also in blood cells suspensions (Pettitt et al. 1991). Analysis of the supernatants from challenged cells showed that calcium ionophore alone stimulates PGE_2 production (Knight et al. 1993b; Secombes et al. 1994a; Rowley et al. 1995; Rowley et al. 2012) but also $PGF_2\alpha$ (Pettitt et al. 1991; Rowley et al. 2012) and PGD_2 (Rowley et al. 2012). Interestingly, indomethacin, a COX inhibitor, inhibited PGE generation following ionophore challenge (Secombes et al. 1994a).

Knight and coworkers analyzed the production of PGE by different tissues from rainbow trout upon calcium ionophore treatment (Knight and Rowley 1995). The results showed that the greatest amount of PGE was found in the brain, followed by the heart, spleen and the liver and lower amounts were found in ovary, muscle, eye and skin. Unfortunately, the head kidney, one of the main organs in the immune response in fish, was not included in this study.

4.2.1.3. Modulation of PG production in fish immune cells by PAMPs

However, most studies have concentrated on the biosynthetic capacity for prostaglandin generation by fish leukocytes challenged in vitro with different PAMPs, including LPS (Rowley et al. 1995; Zou et al. 1999; Holen et al. 2012a; Boltana et al. 2014b), Poly I:C (Polyinosinic-polycytidylic acid, a synthetic analog of double-stranded RNA, a molecular pattern associated with viral infection) (Holen et al. 2012a), β-glucans (Knight et al. 1993b; Paramá et al. 2007; Rowley et al. 2012), peptidoglycan (PGN) (Boltaña et al. 2011) and bacterial DNA (Table 1).

Several reports exist on the ability of LPS, from different sources, to modulate PGE production by immune cells in different fish species like trout (Rowley et al. 1995), goldfish (*Carassius auratus*) (Zou et al. 1999), gilthead seabream (Boltana et al. 2014b) and Atlantic cod (Holen et al. 2012a). For example, Holen and coworkers showed that neither poly I:C nor LPS alone is able to increase PGE_2 production by head kidney leukocytes in Atlantic cod (Holen et al. 2012a). Similar results has been described for trout head kidney macrophages stimulated with *Escherichia coli* 011:B4 LPS, although, in this case, LPS exerted a synergistic effect with calcium ionophore on PGE_2 generation (Rowley et al. 1995). In contrast, LPS from *V. anguillarum*, *Aeromonas salmonicida* or *E.coli*, was able to increase the production of PGE_2 by gilthead seabream macrophages at the highest doses tested, such an increase being correlated with the upregulation of COX-2 expression (Boltana et al. 2014b). An additional study, performed by Zou and coworkers, showed that treatment of goldfish macrophages (head kidney

macrophage cell line or primary macrophages) with *Salmonella enterica* serovar Typhimurium LPS before challenge with calcium ionophore increased PGE_2 generation by these cells (Zou et al. 1999). In addition, LPS-mediated increased PGE generation was abolished in the presence of specific COX-2 inhibitors, while COX-1 inhibitors exerted less effect. All of these studies revealed that there are differences in the effect of LPS in the regulation of PGE_2 production, the exact effect depending on its origin, the doses tested, the fish species and probably the experimental design itself. It is important to note that all these studies were not performed with ultrapure LPS, therefore some of the effects observed could have been mediated by contaminants present in the LPS preparations (Sepulcre et al. 2009).

In regards to β-glucans, studies have been performed using rainbow trout macrophages (Knight et al. 1993b; Rowley et al. 2012) and turbot kidney leukocytes (Paramá et al. 2007). Knight and coworkers, reported that the incubation of rainbow trout macrophages with opsonized and non-opsonized zymosan for 24 h, stimulated PGE_2 production (Knight et al. 1993b). A more recent report studied in depth the increased production of PGE by trout macrophages challenged with opsonized zymosan (Rowley et al. 2012). The analytical technique used in this study did not differentiate between PGE_2 and PGE_3, which the authors refer to as PGE. The study revealed that incubation of the cells for 30 min with zymosan pre-incubated in normal trout serum resulted in greater PGE production compared with zymosan pre-incubated with heat-inactivated trout serum. These results suggest that the complement fragments present in trout serum are responsible for any difference in the level of PGE production between the treatments mentioned. However, the incubation of trout macrophages with zymosan-activated trout serum, which contains complement fragments, did not elicit any induction. Additional experiments in this study showed that short-term incubation of trout macrophages with the fragment of the complement C3a-1 did not induce the generation of PGE but long-term incubation (24 h) resulted in a significant increase in PGE production (Rowley et al. 2012). Parama and coworkers showed that stimulation of turbot kidney leukocytes with β-glucans for 24 h increased the production of PGE_2, while prostaglandin production was blocked by indomethacin (Paramá et al. 2007).

Only one report exists on the ability of peptidoglycan as a stimulant of PG production. In vitro stimulation of trout macrophages with PGN from different *E.coli* serotypes resulted in increased PGE_2 production in a time-dependent manner and a weaker increase in PGD_2 secretion into the culture medium (Boltaña et al. 2011).

All of these studies revealed that, like in mammals (Calder 2008), activation of PRR in fish leukocytes triggers PG production, mainly PGE_2. It should be pointed out that all of these studies have been focused, mainly, on hematopoietic kidney undifferentiated macrophages. In this context, previous studies in mammals have shown differences in the amount and mechanism of PG production among

monocytes and differentiated macrophages in vitro (Bell et al. 1992). However, whether fish tissue macrophages trigger PG production has not been addressed yet.

4.2.2. The in vivo approach

4.2.2.1. Effect of dietary phospholipids on prostaglandin production

It has been well established the relationship between the fatty acid composition of immune cells and their functions (Calder 2008). Fatty acids fulfill a variety of roles within immune cells. Cell membrane phospholipid fatty acids, as precursors for the synthesis of bioactive lipid mediators like PGs, regulate the immune response through the production of eicosanoids (Calder 2008). The fatty acid composition of cells and PG production can be modified by altering oral intakes of certain fatty acids. The use of vegetable oils in fish diets has been extensively studied; and recent work has focused attention on replacing fish oil with alternative fatty acid sources, like plant-derived oils, and their effect on the immune system. The effect of oil diet component on phospholipid fatty acids compositions of different organs and PG production has been documented in several fish species (Bell et al. 1992; Bell et al. 1994a; Bell et al. 1994b; Bell et al. 1995; Bell et al. 1996; Henderson et al. 1996; Bell et al. 1998; Ganga et al. 2005). However, few studies have been conducted on fish leukocytes fatty acid composition and PG production by those cells. For example, a study performed in Atlantic salmon by Bell and coworkers showed that increased linoleic acid intake resulted in a higher incorporation of linoleic acid and in a higher proportion of AA, compared with EPA ones, into membrane phospholipids of leukocytes, which resulted in changes in plasma eicosanoid production profiles (Bell et al. 1992). In a similar way, a study performed in gilthead seabream by Ganga and coworkers showed that substitution of fish oil by vegetable oils rich in oleic, linoleic and linolenic acids in diets, affected fatty acid composition of blood leukocytes by reducing the EPA contents and slightly the AA ones (Ganga et al. 2005). However, while the concentration of plasma PGE_2 was not affected, there was a correlation between plasma PGE_3 and EPA concentration (Ganga et al. 2005). All these studies reveal the impact of different oil sources of fish diets on PG production, an issue to take into account especially for aquaculture. However, more studies are needed to correlate this effect on PG production and the immunological status of fish.

4.2.2.2. Prostaglandin production after immunological challenge

The most common experimental approach for studying the in vivo production of PGs by fish immune cells involves the intraperitoneal injection of microorganisms or microbial products (Rowley 1996). The relatively little work done in this area has been performed in trout (Rowley 1996), turbot (*Scophthalmus maximus* L.) (Paramá et al. 2007) and gilthead seabream (Boltana et al. 2014b) (Table 1). Intraperitoneal injection of zymosan into trout resulted in increased PGE_2 levels in peritoneal exudates compared with fish injected with saline buffer (Rowley 1996). In addition, biopsies of kidney from rainbow trout infected with proliferative kidney disease, the causative agent, showed increased

levels of PGE_2 compared with kidney from non-infected trout (Rowley 1996). This increase in PGE_2 production in vivo may suggests a role for this compound in inflammation, although it could also be argued that it reflects an increase in leukocyte numbers in this tissue (Rowley 1996). Additional work is needed to determine the role of this compound in inflammation.

In a more recent report, Boltaña and colleagues analyzed the production of PGE_2 in plasma from seabream exposed to a dose and time-related challenge with LPS from several bacteria (Boltana et al. 2014b). The results showed that LPS increased the levels of PGE_2 released in a dose-dependent manner, although the levels decreased in a time-dependent manner from 3 to 24 hours post LPS injection.

4.3. Immunoregulatory activities of prostaglandins in fish

4.3.1. The in vitro approach

Several reports exist about the effect of PGs on immune relevant activities in fish (Table 2). Most of them have focused on the biological activity of PGE mainly in trout (Knight et al. 1993b; Secombes et al. 1994a; Knight and Rowley 1995; Hill et al. 1999; Secombes et al. 2001a; Belmonte et al. 2014b) but also in Atlantic salmon (Fast et al. 2005a), sticklebacks (*Gasterosteus aculeatus*) (Kutyrev et al. 2014b) and turbot (Paramá et al. 2007). Little is known about the effect of other prostaglandins, like PGD_2 and its cyPGs derivates, on fish immune activities. The most common experimental design used in these studies is to administer prostaglandins or inhibitors of the same to fish leukocytes in vitro. This approach has the problem of prostaglandin stability, since bioactive PGs are rapidly converted to inactive metabolites. For example, PGE_2 has a half-life of 30 s in the circulatory system (Fitzpatrick et al. 1980a) and 26 h in culture media (Watzer et al. 2009b). Therefore, some of these studies were performed using metabolically stable synthetic analogs, such as 16,16-dimethyl-PGE_2 (diMePGE_2) for PGE_2 assays (Knight and Rowley 1995; Novoa et al. 1996a).

Biological activity	PG	Fish	Experimental conditions	References
The *in vitro* approach				
Inhibition of the proliferative response	PGE_2, PGE_3	Trout	Total HK leukocytes exposed to PHA-P	(Secombes et al. 1994a)
Inhibition of PFC formation	$diMePGE_2$, PGE_2, PGE_3	Trout	Splenocytes from immunized fish	(Knight and Rowley 1995)
Potentiation of the phagocytic response	PGE_2	Trout	Yeast uptake by HK macrophages	(Knight et al. 1993b)
Up-regulation of IL-6, IL-10 and COX-2, and downregulation of IFNγ and TNFα.	PGE_2	Trout	Total HK leukocytes exposed or not to *S.parasitica* cell wall carbohydrates	(Belmonte et al. 2014b)
Inhibition of MHCII expression	PGE_2	Trout	Total HK leukocytes	(Secombes et al. 2001a)
Inhibition of cell aggregation	PGE_2	Trout	Thrombocytes treated with U-46619 and trout fibrinogen	(Hill et al. 1999)
Inhibition of ROS production	$diMePGE_2$	Trout	HK macrophages pre-stimulated with LPS, glucan or MAF	(Novoa et al. 1996a)
Inhibition of the expression of MHCI, MHCII and IL-1β and regulation of COX-2 and TNFα	PGE_2	Atlantic salmon	Macrophage-like SHK-1 cells treated or not with LPS	(Fast et al. 2005a)
Modulation of the respiratory burst, cell viability and leukocyte subpopulations	PGE_2	Sticklebacks	Short- and long-term head kidney leukocytes cultures derived from infected or not fish	(Kutyrev et al. 2014b)
Induction *vs* inhibition of ROS production	PGE_2	Turbot	Pronephric leukocytes exposed to *P.dicentrarchi* cysteine proteinases	(Paramá et al. 2007)
The *in vivo* approach				
Depression in antibody titers	PGE_2	Trout	Immunization with *A.salmonicida*	(Knight and Rowley 1995)

Table 2. Immunoregulatory activity of prostaglandins in fish

In mammals, the effect of PGE_2 in phagocytes is usually anti-inflammatory. Indeed, PGE_2 has been demonstrated to inhibit the production of proinflammatory molecules and increase the secretion of anti-inflammatory cytokines, such as IL-10, and potently inhibit LPS-induced TNF-α (Medeiros et al. 2012). In fish, a broad range of trout leukocyte activities has been seen to be modulated by PGE. For example, Secombes and coworkers demonstrated that PGE_2 and, to a lesser extent PGE_3, exert an inhibitory effect on the proliferative response of rainbow trout leukocytes exposed to phytohaemaglutinin-P (Secombes et al. 1994a). The exogenous addition of PGE_2 induced the expression of cytokines like IL-6 and IL-10 in trout head kidney leukocytes but suppressed the expression of TNFα, the membrane IgM, the membrane and soluble IgT and genes involved in cellular immunity like INFγ and the gamma interferon induced protein (Belmonte et al. 2014b). In addition, PGE_2 could regulate its own production by the induction of COX-2 (Belmonte et al. 2014b). PGE_2 also inhibited the expression of the major histocompatibility complex II (MHCII) in trout head kidney leukocytes (Secombes et al. 2001a). In a similar study performed in Atlantic salmon macrophage-like cell line derived from head kidney (SHK-1), PGE_2 reduced the LPS-induced expression of MHCI and MHCII in both LPS-stimulated and non-stimulated controls (Fast et al. 2005a). In addition, COX-2 expression increased in

LPS-stimulated cells exposed to PGE_2 for 4 but decreased in 24 h exposed cells. In contrast, PGE_2 increased the expression of TNFα (Fast et al. 2005a).

Another function regulated by the presence of PGE is the capacity of splenocytes from immunized trout to generate plaque-forming cells (PFC) challenged with the immunogen (Knight and Rowley 1995). It is important to note that in the same study PGE_2, PGE_3 and $diMePGE_2$ exerted similar effects at the same doses. In addition, the presence of the COX inhibitor indomethacin caused a significant increase in PFC numbers.

Studies performed with trout macrophages have shown that PGE_2 modulates some of their activities, for example it increases their phagocytic response, causing a significant dose-dependent enhancement of yeast uptake compared with non-treated cells (Knight et al. 1993b). Another function modulated by PGE_2 in trout macrophages is reactive oxygen species (ROS) production. In this regard, an elegant study performed by Novoa and coworkers showed that $diMePGE_2$ inhibits the respiratory burst activity of stimulated macrophages either by co-stimulation with different stimuli or using previously stimulated macrophages (Novoa et al. 1996a). In a similar way, PGE_2 reduced ROS production in both head kidney leukocytes from non-infected and *Schitocephalus solidus*-infected sticklebacks (Kutyrev et al. 2014b). An additional work performed in turbot pronephric leukocytes showed that PGE_2 at the lower doses tested increased the respiratory burst induced by the *Philasterides dicentrarchi* proteinases (Paramá et al. 2007). In contrast, the highest doses used suppressed the increase in superoxide anion.

In vitro systems are convenient for studying the role of PGs in regulating the main immune activities in different fish species. Of the few studies available, some of them point to opposite effects of PGE in the regulation of immune cell activity, depending on the cell type and the fish species. It is important to highlight the heterogeneity of the different approaches described; for example, the effective dose of PGE_2 in all these studies varied from 0.17 nM (Kutyrev et al. 2014b) to 10 µM (Secombes et al. 2001a) and the time of incubation with PGE varied from 1h (Knight et al. 1993b; Paramá et al. 2007) to 48h (Novoa et al. 1996a; Fast et al. 2005a), while the state of cell activation also varied: challenged (Novoa et al. 1996a; Fast et al. 2005a; Belmonte et al. 2014b) or non-challenged cells (Knight et al. 1993b; Secombes et al. 2001b). Another important variant to take into consideration in the different studies is the amount of serum present in the culture media. It has been described that albumin, the main component of serum, reduces PGE_2 bioavailability, and appears to play a role in modulating PGE_2-mediated immune dysfunction (O'brien et al. 2014). So, additional work is needed to increase our knowledge of the role of PGE in the fish immune response and the environmental factors that may influence the heterogeneity in the results observed across teleost fish species.

Little is known about the effect of other prostaglandins like PGD_2 or its CyPGs derivates on immune relevant activities in fish.

4.3.2. The in vivo approach

Only one study exists on the role of PGs in modulating in vivo the immune activities in fish (Table 2) (Knight and Rowley 1995). In this study, Rowley and coworkers analyzed the dynamics of antibody formation in serum from immunized rainbow trout with formalin-inactivated *A. salmonicida* in the presence or absence of several doses of the stable PGE_2 analog, diMePGE_2. The results showed significant depression in the antibody titers from fish injected with the higher doses tested showed compared with those animals immunized with bacteria alone (Knight and Rowley 1995). Additional roles for PGs in vivo are at present unknown but should be the subject of further studies.

4.4. Summary and future perspectives

PGs are key components of the immune system. The multiple effects they exert on immune cells depend on their individual concentrations and the combinatorial effects of the factors present in a specific context. In vitro and in vivo biosynthesis of prostaglandins has been studied in fish (Table 1). The determination of PG production by fish leukocytes is affected by their low stability, a problem which different methods have tried to overcome, although each method also has its own limitations. It is important to stress that all the in vitro determinations were performed in supernatants from culture cells, considering the PGs as part of the secretome. However, no work has been done on PG quantification inside the cells in fish. In addition, in vitro and in vivo functions of prostaglandins have been studied in fish (Table 2). Some of the prostaglandins generated by fish leukocytes have been reported to be involved in inflammatory responses in several fish species. The variety of effects that they can elicit could reflect the presence of specific PG receptors in many cells types. However, no work has been done in this regard either. The continued improvements made in analytical techniques, as well as the increased availability of specific metabolically stable synthetic analogs for PGs and of receptor agonists and antagonists, should throw more light on their role in the regulation of fish immunity in forthcoming years.

OBJECTIVES

This work has the following specific objectives:

1. To determine the molecular characterization of prostaglandin D synthase (PGDS) of gilthead seabream (*Sparus aurata*, L.).

2. To study the expression profile of genes involved in prostaglandin metabolism and their modulation in vitro and in vivo under challenge conditions in gilthead seabream (*Sparus aurata*, L.).

3. To analyze the role of different prostaglandins (PGE_2, PGD_2, $15dPGJ_2$ and Δ^{12}-PGJ_2) in the main biological activities of gilthead seabream phagocytic cells (macrophages and acidophilic granulocytes).

4. To determine the signaling pathways involved in PGE_2 and $15dPGJ_2$ activities in macrophages and acidophilic granulocytes of gilthead seabream (*Sparus aurata*, L.), respectively.

CHAPTER I: Professional phagocytic granulocyte-derived PGD_2 regulates the resolution of inflammation in fish

Abstract

Prostaglandins (PGs) play a key role in the development on the immune response, through the regulation of both pro- and anti-inflammatory processes. PGD_2 can be either pro- or anti-inflammatory depending on the inflammatory milieu. Prostaglandin D synthase (PGDS) is the enzyme responsible for the conversion of PGH_2 to PGD_2. In mammals, two types of PGDS synthase have been described, the hematopoietic (H-PGDS) and the lipocalin (L-PGDS). In the present study we describe the existence of two orthologues of the mammalian L-PGDS (PGDS1 and PGDS2) in the gilthead seabream and characterize their gene expression profiles and biological activity. The results showed a dramatic induction of the gene coding for PGDS1 in acidophilic granulocytes (AGs), which are functionally equivalent to mammalian neutrophils, after a prolonged *in vitro* activation with different pathogen associated molecular patterns (PAMPs). In contrast PGDS2 was not expressed in these cells. The functional relevance of the induction of PGDS1 in AGs was confirmed by the ability of these cells to release PGD_2 upon PAMP stimulation. To gain further insights into the role of PGD_2 in the resolution of inflammation in fish, we examine the ability of PGD_2 or its cyclopentenona derivates (cyPGs) to modulate the main functional activities of AGs. It was found that both PGD_2 and cyPGs inhibited the production of reactive oxygen species and downregulated the transcript levels of the gene encoding interleukin-1β. Altogether, these results demonstrate that the use of PGD_2 and its metabolites in the resolution of inflammation was established before the divergence of fish from tetrapods more than 450 million years ago and support a critical role for granulocytes in the resolution of inflammation in vertebrates.

1. Introduction

Eicosanoids, including prostaglandins (PGs), leukotrienes and lipoxins are small fatty acid molecules derived from polyunsaturated fatty acids released from the cell membrane phospholipids, mainly arachidonic acid. Prostaglandins play a significant role in the progress of the immune response at multiple levels, often in concert with microbial ligands, cytokines, chemokines, and various other factors (Hirata and Narumiya 2012a).

The first enzyme involved in PG biosynthesis is the cyclooxygenase (COX) which converts arachidonic acid to PGH_2. There are several forms of COX: COX-1 is constitutively expressed in nearly all cell types, while COX-2 is mainly inducible in inflammatory conditions and is expressed by a more limited range of cell types (Simmons et al. 2004a). PGH_2 is subjected to further conversion, leading to the generatio of PGs generation including PGE_2 and PGD_2 (Joo and Sadikot 2012a). PGD_2 is converted to cyPGs, i.e. 15-deoxy-$\Delta^{12,14}$ PGJ_2 and $\Delta^{12}PGJ_2$, by albumin-independent and -dependent reactions, respectively (Shibata et al. 2002a).

PGD_2 synthase is responsible for production of PGD_2 (the major prostaglandin in the central nervous system and in immune cells) from PGH_2. In mammals, two distinct types of PGD_2 synthase have been identified: the hematopoietic form (H-PGDS) and the lipophilic ligand-carrier protein (lipocalin)-type enzyme (L-PGDS). These two enzymes share no homology in DNA or polypeptide sequences (Kanaoka et al. 1997b) and show different tissue expression profiles, L-PGDS being mainly expressed in the central nervous system (CNS) and related organs (Urade and Hayaishi 2000) and H-PGDS in hematopoietic cells, including macrophages (Urade and Eguchi 2002). Previous studies have reported an important role for H-PGDS in the onset and resolution of acute inflammation (Rajakariar et al. 2007a). However, while H-PGDS is constitutively expressed in macrophages, L-PGDS is not detected in macrophages in normal conditions but is only expressed in a pathological environment (Cipollone et al. 2004). The production of PGD_2 by neutrophils is a controversial topic. Pouliot and coworkers reported that the main PGH_2–derived metabolites produced *in vitro* by human neutrophils following activation by a variety of inflammatory stimuli were TXB_2 and PGE_2, whereas no detectable level of PGD_2 was observed (Pouliot et al. 1998). However, two more recent studies have identified the production of PGD_2 by human neutrophils during spontaneous apoptosis (Brown et al. 2003) and by mouse neutrophils during acute lung injury (Murata et al. 2013). Strikingly, this last mentioned paper showed that H-PGDS was responsible for the production of PGD_2 by mouse neutrophils.

Taking into account that fish express several isoforms of L-PGDS, but none of H-PGDS, the aim of this study was to determine the role of PGDSs and their products, PGD_2 and cyPGDs, in the resolution of inflammation in the teleost fish gilthead seabream (*Sparus aurata* L.) and, in particular, the contribution of acidophilic granulocytes (AGs), which are functionally equivalent to mammalian

neutrophils (Sepulcre et al. 2002; Chaves-Pozo et al. 2004; Chaves-Pozo et al. 2005a; Sepulcre et al. 2007a; Sepulcre et al. 2011a; Cabas et al. 2013), to the production of PGD_2.

2. Materials and Methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinoperygii, Sparidae) were bred and kept at the Oceanographic Centre of Murcia (Spain) in a 14 m^3 running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). Fish were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Amino acid sequence analysis

Sequence homology analysis was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the blast tool at Ensembl (Ensembl Genome Browser, http://www.ensembl.org). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/). A direct comparison between two sequences was performed using the EMBOSS Needle program within the EMBL-EBI website, while multiple sequence alignment was carried out with 114 the ClustalX version 2.1 program (Larkin et al. 2007). The domains of the proteins deduced from the nucleotide sequences were determined using the Simple Modular Architecture Research Tool (SMART), from the European Molecular Biology Laboratory (EMBL) website (http://smart.embl-heidelberg.de/) (Schultz et al. 1998; Letunic et al. 2012).

2.3. Isolation of phagocytes

AGs were obtained by MACS as described earlier (Roca et al. 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) (Sepulcre et al. 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100-200 µl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al. 2006).

2.4. Cell culture and treatments

Isolated AGs were stimulated at 23°C with 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (*Va*DNA) (Pelegrin et al. 2004) or 100 ng/ml flagellin (Invivogen) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 μg/ml strepomycin (Biochrom). These concentrations of PAMPs have been found to be optimal for *in vitro* activation of seabream AGs (Sepulcre et al. 2007a). AGs were cultured with or without the synthetic stable analog of PGD_2 (5-20 μM, 16,16-dimethyl PGD_2), the synthetic stable analog of 13,14-dihydro-15-keto PGD_2 (5-20 μM, 11-deoxy-11-methylene-15-keto PGD_2), 15-deoxy-$\Delta^{12,14}$ PGJ_2 (0.5-1 μM) and $\Delta^{12}PGJ_2$ (1-10 μM) (Cayman Chemical) (Ward et al. 2002).

2.5. In vivo sampling and experimental infections

Fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10^8) of exponentially growing *V. anguillarum* R82 cells (Chaves-Pozo et al. 2004). Head kidney, spleen, thymus, liver, gills, blood and peritoneal exudates cells were obtained 4 h after bacterial challenge. Gut and brain were obtained from PBS treated fishes. All samples were processed for subsequent real-time RT-PCR (see below).

2.6. Analysis of gene expression

Total RNA was extracted as indicated above and treated with DNase I, Amplification grade (1 unit/μg RNA, Life technologies). The SuperScrip III RNase H^- ReverseTranscripase (Life technologies) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1 μg of total RNA at 50 ºC for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the ribosomal protein S18 (*rps18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ for the constitutive expression analysis). The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice.

Gene	Accesion number	Name	Primer sequence (5'-3')
pgds1	AM971774	F	CACGCCATAACATGGTGAAG
		R	GACCGTAAAGTGCCACCTGT
Pgds2	AM959591	F	GTGCTGGGAGCCGTGCTCTG
		R	GCCGTGCCCACCTTCATGCT
ptgs2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
Il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
rps18	AY587263	F1	AGGGTGTTGGCAGACGTTAC
		R1	CTTCTGCCTGTTGAGGAACC

Table 1. Gene accession numbers and primer sequences used for gene expression analysis.

2.7. Measurement of PGD2 by EIA

PGD_2 synthesis was analyzed in the supernatants from AGs stimulated or not with *Va*DNA for one to 3 days using a commercial enzyme immunoassay (EIA) kit (Cayman Chemical).

2.8. Cell viability

Aliquots of cell suspensions were diluted in 200 µl PBS containing 40 µg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by flow cytometry.

2.9. Respiratory burst assays

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by AGs after different stimulation times (Mulero et al. 2001). This was done by adding 100 µM luminol and 1 µg/ml PMA (both from Sigma-Aldrich), while the chemiluminescence was recorded every 117 seconds for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of quadruple readings, expressed as the slope of the reaction curve from 117 to 1170 seconds, from which the apparatus background was subtracted.

2.10. Statistical analysis

Data were analyzed by ANOVA and a Tukey's multiple range test to determine differences between groups.

3. Results

3.1. Characterization of genes encoding PGDS in gilthead seabream

Searches within the publicly available EST database of the European Nucleotide Archive (ENA) allowed us to identify two genes encoding for gilthead seabream, PGDS1 and PGDS2 (ENA accession numbers **AM975584** and **AM959591**, respectively) (Figure 1A and 1B). An interesting difference between them was the presence of two ATTTA motifs in the 3' untranslated region (UTR) of the PGDS1. These motifs have been shown to be responsible for the instability of mammalian (Han et al. 1990) and fish (Roca et al. 2007) cytokine mRNAs. Both cDNAs encoded a single open reading frame, which translated into putative 184 and 180 amino acid polypeptides, showing 42.8% (PGDS1) and 46.1% (PGDS2) amino acid similarity to human L-PGDS (Figure 2A). In addition, the presence of a lipocalin domain in both seabream PGDS proteins further confirmed that they are PGDS of the lipocalin type (Figure 2B). Previous studies showed that non-mammalian (zebrafish, xenopus and chicken) and mammalian L-PGDS formed an "L-PGDS sub-family" in the lipocalin gene family (Fujimori et al. 2006). One of the features of this family is the presence of conserved folding patterns and three structurally conserved regions (SCRs) (Flower 1993). Strikingly, however, the primary structure of zebrafish, rainbow trout and seabream L-PGDS lacked the essential cysteine residue required for PGDS activity found in mammalian L-PGDS (Urade et al. 1995; Bayne et al. 2001b; Fujimori et al. 2006).

A

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acacatcctcaacacttggccactgttccccggtccctagcgatgattggctgacaaccc
agtgggtggatcaggagaaaaacaaacattgccccaggccttgccactctgtacctgtat
cggctgactgcctactaacagttcacttcaggctcgggtcagataccctgtctcaacctt
tttttggatccgttcacctggcctcttcttttctcagcc ATG AGGACCACTATGGTCGCC
                                          M  R  T  T  M  V  A
GTTGTCATGGTGATGTGCGCGATGATGGCACACGCAGACGTGAAGCCACAGAGAGATTTC
 V  V  M  V  M  C  A  M  M  A  H  A  D  V  K  P  Q  R  D  F
AACCTGCAGAGGTTTGCAGGGAGATGGTACAGAGTGGGCCTGGCCTATGACTCCCCAAAC
 N  L  Q  R  F  A  G  R  W  Y  R  V  G  L  A  Y  D  S  P  N
TTTGTCCCCTACAGAGACAAAGTAAAGGTCTCCATGGGCGTCATCACAGTGCTGCCGAGC
 F  V  P  Y  R  D  K  V  K  V  S  M  G  V  I  T  V  L  P  S
GGCAACGTCAACCTCACAATGTGGGACGCCACACCTGTCGGCTGTCTCAGCAAGGTGTAC
 G  N  V  N  L  T  M  W  D  A  T  P  V  G  C  L  S  K  V  Y
CAGTATGAGAAGACCAACGTGGCCGGACAGTTCACCTACTTCAGCACACGCCATAACATG
 Q  Y  E  K  T  N  V  A  G  Q  F  T  Y  F  S  T  R  H  N  M
GTGAAGGACATCACAGTGGTCGACACAAACTACACTGATTACGCTTTGGTTCTCAAACAC
 V  K  D  I  T  V  V  D  T  N  Y  T  D  Y  A  L  V  L  K  H
AAGGTTTTAACCGAGAGTACACACAGGTGGCACTTTACGGTCGCTCTCAAAGCCTCAGA
 K  V  F  N  R  E  Y  T  Q  V  A  L  Y  G  R  S  Q  S  L  R
AACAATGTTATCCAGAAGTTTAAAGCCTTCGCCTTGTCCCAGGGTTTCTCCAAAGAGTCT
 N  N  V  I  Q  K  F  K  A  F  A  L  S  Q  G  F  S  K  E  S
ATTCTAACTCCACCCCCTGCAGAAAATTGCCCTCCATCAGGATCTGGACGT TAG GTTCCC
 I  L  T  P  P  P  A  E  N  C  P  P  S  G  S  G  R  -
tgacgagcctgagatgaggctggcagcgtactcttaaaaagctctttctctgcttcgcac
ttcctttgaagaactacaaagcggaactctcttcagcaaatatcatcgttgtggctacaa
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cgttcc atttta gtcctgctgtgttgtcatagttaaatgaacaatatgcacctgaagctt a
ttta aaccctttctctttcttcccttgatcaccaaacaccaaagaaaatgttttaaagaa
aaaagcttttttgttgtggtggctgtttttttgtctctca ataaa ttgttgaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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B

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ctaccatccacacacttctccaatcgtcgttcctgcgactgatcagtagct ATG ACTTTA
                                                      M  T  L
CTGCTGACGGTGCTGGGAGCCGTGCTCTGCTCCTGGACGGCTTCTTCTGAAGTCGTACCA
 L  L  T  V  L  G  A  V  L  C  S  W  T  A  S  S  E  V  V  P
CAGGCAGATTTTAATCCACAGTCGATGGCAGGGAAGTGGTACCTGGTTGGATTCGCCACC
 Q  A  D  F  N  P  Q  S  M  A  G  K  W  Y  L  V  G  F  A  T
AATGCCCAGTGGTTTGTCAACCGTAGAGACAGCATGAAGGTGGGCACGGCCATGTTCACC
 N  A  Q  W  F  V  N  R  R  D  S  M  K  V  G  T  A  M  F  T
CCAACTGCAGACGGGGACCTGGACCTCTCATACGCCAGTCTCAACTCTGATGGCTCTTGC
 P  T  A  D  G  D  L  D  L  S  Y  A  S  L  N  S  D  G  S  C
TGGAGACTGAACAACCTGGCCAAGAAGATTGACATGCCTGGAAAGTTCACGTACACAAGC
 W  R  L  N  N  L  A  K  K  I  D  M  P  G  K  F  T  Y  T  S
TGGGGGAACGAGAACGACATGCGCAAGGTGGATGTGAAGTACGACGAGTACGCCGTCATT
 W  G  N  E  N  D  M  R  K  V  D  V  K  Y  D  E  Y  A  V  I
CACACCATTAAGACCAAGGGCGGTGTCATCACTGTTGTCAACAAACTTTATGGCCGTAGC
 H  T  I  K  T  K  G  G  V  I  T  V  V  N  K  L  Y  G  R  S
ATGGACCTCAGCGCTGACCTGCTGGAGAAGTTCAGGCAGTTCTCCTTGGAGACTGGGGTC
 M  D  L  S  A  D  L  L  E  K  F  R  Q  F  S  L  E  T  G  V
CTGCCTGAAAACATCGCTTTCCTTCCCAGAAATGCGGAGTGCCCAGCTGCC TAG tttctg
 L  P  E  N  I  A  F  L  P  R  N  A  E  C  P  A  A  -
actcctcatgcgtcaatgatgctgtctgaagacctgaatgcttgtcgccagcagtctgca
tcaaggtctttgccataaactgaacaaatgaatacaaaaggacttctgtttatttcctct
gctctttcagatgccactcgacgtgaagtaaaagcctggtttcaatatacaatactttgc
gtttatctatcaaccagccttcaaagattcccttcagacatgttttgaga ataaa acct
tctactttcagtaga
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Figure 1. Nucleotide sequences and deduced amino acid sequences of seabream PGDSs**.** PGDS1 (A) and PGDS2 (B). The open reading frames are in uppercase letters**.** The start codon (ATG) and the stop codon (TGA) are in bold. The motif associated with mRNA instability (ATTTA) is in bold and underlined. The polyadenylation signal (AATAAA) is in bold and italics.

A

```
zfPGDSb     ----MTSVVVKMLCLLLCAVFAS--ADVMPMTDFDLQKVEGKWYLVGFATNAKWFVSHKD 54
sbPGDS2     ----MT-LLLTVLGAVLCSWTAS--SEVVPQADFNPQSMAGKWYLVGFATNAQWFVNRRD 53
sbPGDS1     -----MRTTMVAVVMVMCAMMAH--ADVKPQRDFNLQRFAGRWYRVGLAYDSPNFVPYRD 53
hPGDS       MATHHTLWMGLALLGVLGDLQAAPEAQVSVQPNFQQDKFLGRWFSAGLASNSSWLREKKA 60
                       :  ::    *   ::*    :*: : . *:*: .*:* ::  :   :

zfPGDSb     DMKMGTAMLVPTQEGDLDLSYSNLKSDGSCWRMTYLAKKTETPGRFVFYSQRWGNDNDMR 114
sbPGDS2     SMKVGTAMFTPTADGDLDLSYASLNSDGSCWRLNNLAKKIDMPGKFTYTS--WGNENDMR 111
sbPGDS1     KVKVSMGVITVLPSGNVNLTMWDATPVG-CLSKVYQYEKTNVAGQFTYFSTRHNMVKDIT 112
hPGDS       ALSMCKSVVAPATDGGLNLTSTFLRKNQ-CETRTMLLQPAGSLGSYSYRSPHWGSTYSVS 119
             :.:  .:..   .*.::*:         *       :     * : : *   .   .:

zfPGDSb     VVDAKFDEYAIFHTIKTKG-GVSEILNKLYSRTPEMVDDLKEKFRQFCLDTGILEENIVM 173
sbPGDS2     KVDVKYDEYAVIHTIKTKG-GVITVVNKLYGRSMDLSADLLEKFRQFSLETGVLPENIAF 170
sbPGDS1     VVDTNYTDYALVLKHKVFN-REYTQV-ALYGRSQSLRNNVIQKFKAFALSQGFSKESILT 170
hPGDS       VVETDYDQYALLYSQGSKGPGEDFRMATLYSRTQTPRAELKEKFTAFCKAQGFTEDTIVF 179
             *:..: :**:. .    .      :  **.*:     :: :**  *.   *.  :.*

zfPGDSb     LPQNGECSVAA--- 184
sbPGDS2     LPRNAECPAA---- 180
sbPGDS1     PPPAENCPPSGSGR 184
hPGDS       LPQTDKCMTEQ--- 190
              *   :*
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B

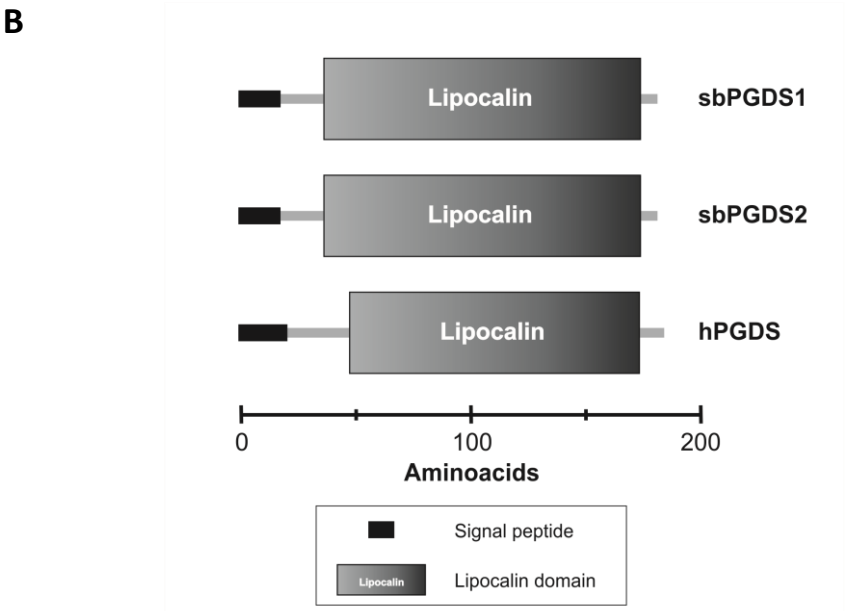

Figure 2. Sequence alignment and molecular characteristics of seabream PGDSs. (A) Amino acid sequences predicted from seabream L-PGDS cDNAs (sbPGDS1 and sbPGDS2) were compared with those of known zebrafish (*Danio rerio*) PGDSb **(**zfPGDSb, accession number BC116554**)** and human L-PGDS cDNAs **(**hPGDS, accession number NP_000945.3**)**. The Cys residue essential for PGDS activity in mammals are highlighted in grey, and the two Cys residues required for the disulfide-bond formation are in bold. Three SCRs (Structural Conserved Regions) are indicated by closed squares inside the alignment. (B) Schematic representation of seabream and human PGDS domain organization. The signal peptides are shown as black boxes and the lipocalin domains as grey rectangles labeled with the word "LIPOCALIN". The position of each domain is indicated with respect to a rule.

3.2. PGDS1 gene expression is induced upon AGs activation

As the production of PGD_2 by mammalian neutrophils is controversial (Pouliot et al. 1998; Brown et al. 2003; Joo et al. 2007), we analyzed the expression of PGDS1 and PGDS2 by RT-qPCR in seabream AGs, which are functionally equivalent to mammalian neutrophils (Sepulcre et al. 2002; Chaves-Pozo et al. 2004; Chaves-Pozo et al. 2005a; Sepulcre et al. 2007a; Sepulcre et al. 2011a; Cabas et al. 2013), after stimulation *in vitro* with different PAMPs. The results showed that *Va*DNA or flagellin stimulation resulted in a gradual increase of the mRNA levels of PGDS1 with the stimulation time (Figure 3A). Notably, PGDS2 was not expressed in control or stimulated AGs (Figure 3D). It is important to note that the expression of the genes encoding prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2), the limiting enzyme in PG production (Figure 3B), and interleukin-1β (IL-1β) (Figure 3C) also showed a dramatic up-regulation but with different kinetics; peaking at the shortest time analyzed (1 day post-stimulation).

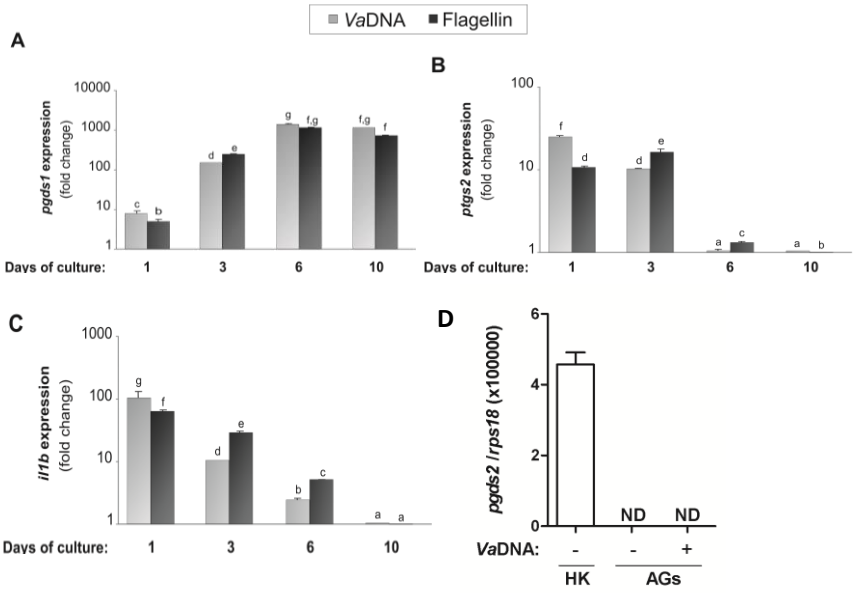

Figure 3. *pgds1* expression is induced upon AGs activation. AGs from head kidney were stimulated for 1, 3, 6 and 10 days with 50 μg/ml *Va*DNA or 0.1μg/ml flagellin in sRPMI supplemented with 5% FCS and the mRNA levels of *pgds1* (A) and *pgts2* (B) and *il1b* (C) were studied by real-time RT-PCR .The gene expression is normalized against *rps18* and expressed as mean±S.E of the gene mRNA fold increase in stimulated cells relative to non cultured cells. Different letters denote statistically significant differences among the groups according to a Tukey test. The groups marked with "a" did not show statistically significant differences from control cells ($p<0.05$). Total head kidney leukocytes or in purified AGs were stimulated or not for 24 hour with 50 μg/ml *Va*DNA in sRPMI supplemented with 5% FCS and the mRNA levels of *pgds2* (D) were studied by real-time RT-PCR. The results are expressed relative to *rps18* and as the mean ± S.E.M. of triplicate. ND, not detected.

3.3. AGs produce PGD2 following stimulation with PAMPs

The expression data obtained in AGs, together with the fact that previous reports have shown that non-mammalian L-PGDSs have no, or very weak, PGDS activity (Fujimori et al. 2006), prompted us to analyze the production of PGD_2 by these cells. The results showed that AGs were able to produce PGD_2 in vitro and that this production increased significantly in response to stimulation with VaDNA (Figure 4).

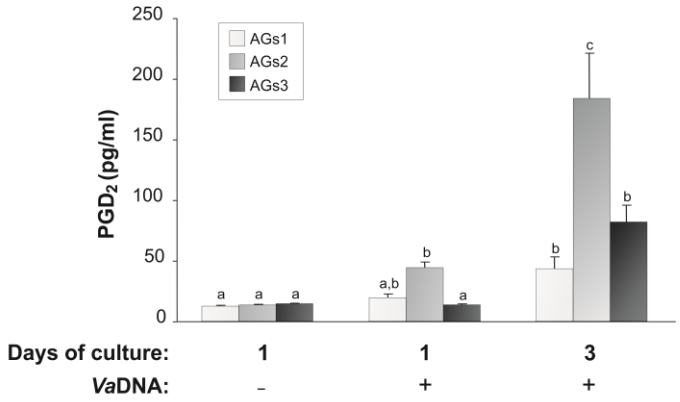

Figure 4. PGD_2 production by AGs upon stimulation. Head kidney AGs from three fish (AGs1, AGs2 and AGs3) were stimulated for 1 and 3 days with 50 μg/ml *Va*DNA in sRPMI supplemented with 5% FCS and the production of PGD2 was analyzed in the supernatants by ELISA. The data are expressed as mean±S.E (n=3). Different letters denote statistically significant differences among the groups according to a Tukey test. The groups marked with "a" did not show statistically significant differences from one day cultured control cells The results are representative of two independent experiments. ($p<0.05$).

3.4. PGD2 and its derivates inhibit AGs functions

We next sought to determine the activity of PGD_2 in the main AGs functions, i.e. the production of reactive oxygen species (ROS) and cytokines. We used 16,16–dimethyl-PGD_2, a metabolically stable synthetic analogue of PGD_2, which signals through DP1 receptor (Gervais et al. 2001), and the chemically stable ligand of 13,14-dihydro-15-keto-PGD_2 of the PGD_2 metabolite 11-deoxy-11-methylene-15-keto-PGD_2, which signals via the chemokine-related CRTH2 receptor (Hirai et al. 2001b). It was found that physiological concentrations of both compounds inhibited in a dose-dependent manner the induction of ROS upon VaDNA stimulation (Figures 5A and 5B), while cell viability was unaffected (data not shown). It is important to note that the effects of 11-deoxy-11-methylene-15-keto-PGD_2 on ROS production was weaker than that of 16,16–dimethyl-PGD_2. Similarly, only 16,16–dimethyl-PGD_2 was able to inhibit the mRNA levels of IL-1β induced in response to VaDNA (Figure 5C). In the view of these results, we also analyzed the effect of the cyPGs, 15-deoxy-$\Delta^{12,14}PGJ_2$ and $\Delta^{12}PGJ_2$, in AGs

activities. Similarly, to PGD_2, both cyPGs decreased ROS production in a dose-dependent manner (Figures 6A and 6B) and downregulated IL-1β expression by AGs (Figure 6C). It is important to note that cyPGs exerted their effects at lower doses and were more potent in comparison with PGD_2. Collectively, these results indicate that PGD_2 and its derivates have a potent anti-inflammatory effect.

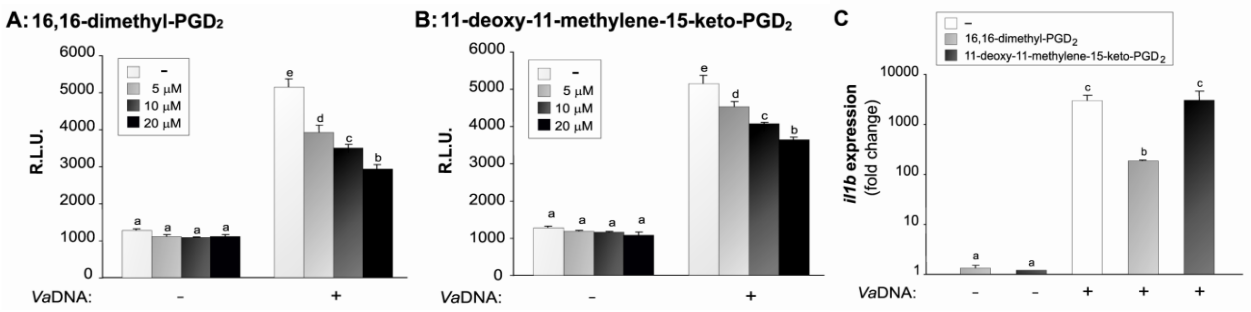

Figure 5. Anti-inflammatory effect of PGD_2 in AGs activities. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of 16,16–dimethyl-PGD_2 (a stable synthetic analog of PGD_2) (A) or 11-deoxy-methylene-15-keto-PGD_2 (a stable synthetic analog of 13,14-dihydro-15-keto-PGD_2) (B), with or without 50 μg/ml of *Va*DNA and their respiratory burst activity was measured. The results are representative of six independent experiments. The mRNA levels of the gene coding for IL-1β were determined by real-time RT-PCR (C). The gene expression is normalized against *rps18* and is shown as relative to the mean of control cells incubated in medium alone. Different letters denote statistically significant differences among the groups according to a Tukey test. The groups marked with "a" did not show statistically significant differences from control cells. The results are representative of three independent experiments. ($p<0.05$).

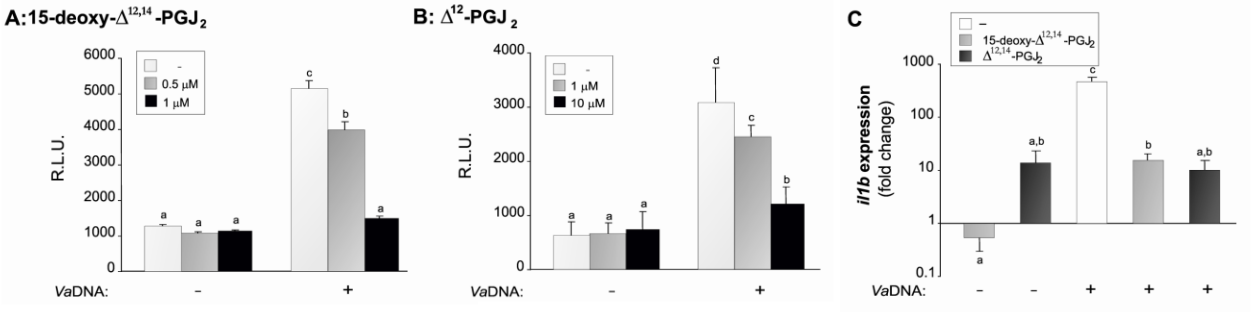

Figure 6. Anti-inflammatory effect of PGJ_2 in AGs activities. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of 15-deoxy-$\Delta^{12,14}$ PGJ_2 (A) or $\Delta^{12}PGJ_2$ (B) with or without 50 μg/ml of *Va*DNA and their respiratory burst activity was measured. The results are representative of six independent experiments. The mRNA levels of the gene coding for IL-1β were determined by real-time RT-PCR (C). The gene expression is normalized against *rps18* and is shown as relative to the mean of control cells incubated in medium alone. The results are representative of two independent experiments. Different letters denote statistically significant differences among the groups according to a Tukey test. The groups marked with "a" did not show statistically significant differences from control cells ($p<0.05$).

3.5. In vivo expression of seabream PGDSs and modulation upon bacterial infection

Analysis of the constitutive expression of seabream PGDS genes showed that both genes were expressed in all the tissues examined, including key immune organs, like head-kidney, spleen, thymus and gills, with the exception of PGDS2 in the gut (Figures 7A and 7B). It is important to note that the highest mRNA levels of PGSD1 were found in liver, while the levels of PGDS2 were much higher in brain, gill and thymus.

To determine whether seabream PGDS might be involved in bacterial infection, healthy seabream juveniles were challenged with the fish pathogenic bacterium *V. anguillarum* and the expression of the genes encoding PTGS2, PGDS1 and PGDS2 was analyzed. Notably, bacterial infection led to an increase in the mRNA levels of PTGS2 and PGSD1 in most of the tissues examined, the blood showing the greatest increase of both genes (Figures 8A and 8B). In contrast, the mRNA levels of PGDS2 increased in blood, spleen and peritoneal exudates, but did not significantly changed in liver, or even decreased (head-kidney, thymus and gill), after the bacterial challenge (Figure 8C). All these data further suggest a role for PGDS1 in the early phases of the resolution of the inflammatory response in gilthead seabream.

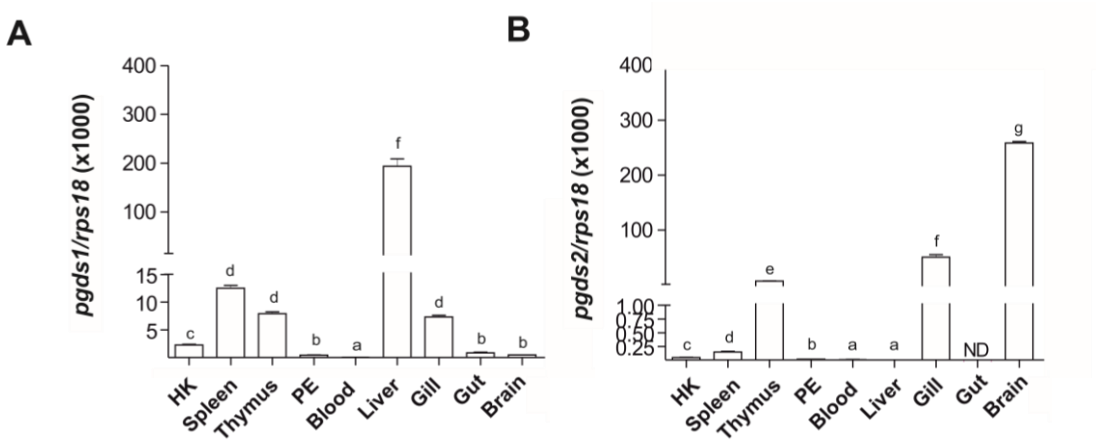

Figure 7. Ubiquitous tissue expression pattern of seabream PGDSs. The mRNA levels of *pgds1* (A) and *pgds2* (B) were determined by real-time RT-PCR in the indicated tissues of control adult specimens. The results are expressed relative to *rps18* and as the mean ± S.E.M. of triplicate Different letters denote statistically significant differences among the groups according to a Tukey test. ND, not detected.

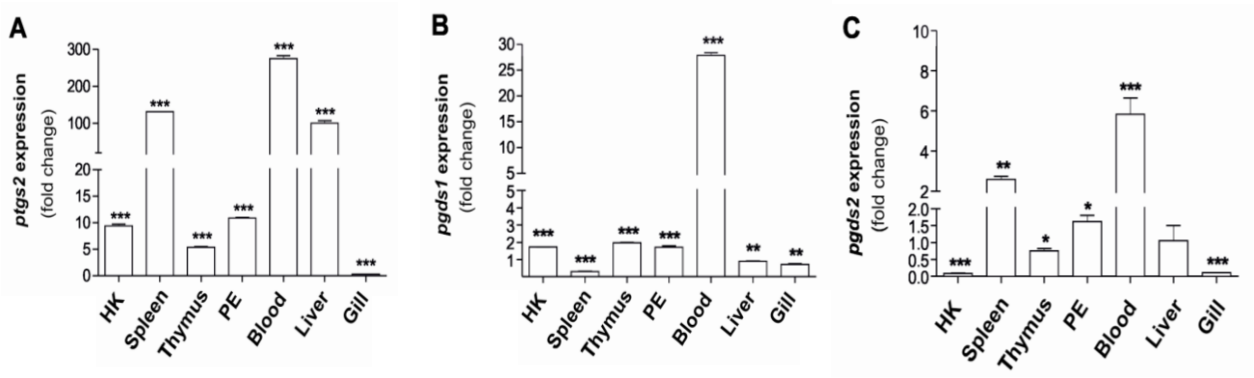

Figure 8. Tissue expression pattern of seabream PG synthesis related genes following infection. The mRNA levels of *pgds1* (A), *pgds2* (B) and *ptgs2* (C) were studied by real-time RT-PCR in the indicated immune of control specimens or following 4 h challenge with 10^8 cells of *Vibrio anguillarum*. The results are expressed as the gene mRNA fold increase in infected fish relative to control fish. The results are presented as mean ± S.E.M. from triplicate samples. Statistically significant differences from non-infected fish according to a Tukey test are shown as: *p<0.05; **p<0.01; ***p<0.001.

4. Discussion

PGDS is the enzyme responsible for the conversion of PGH_2 to PGD_2. Two types of PGDS synthase have been described, the hematopoietic (H-PGDS) and the lipocalin (L-PGDS**)**. This in silico work has revealed the existence of two L-PGDS orthologues in gilthead seabream (PGDS1 and PGDS2). No H-PGDS orthologues have been identified so far in the fish genomes available, including ancient, e.g. the zebrafish (Cypriniformes), and the most evolutionary advanced, e.g. pufferfish (Tetraodontiformes) teleost fish. All these data point to L-PGDS as being responsible for PGD_2 synthesis in gilthead seabream. In addition, the results of the tissue distribution of both L-PGDS genes suggest different roles for these two molecules. In the view of the fact that the highest levels of PGDS2 expression were found in the brain, and that it was weakly modulated after bacterial challenge, and taking into consideration that mammalian L-PGDS is mainly expressed in CNS and related organs (Urade et al. 1993; Taniike et al. 2002), seabream PGDS2 might be considered functionally equivalent to mammalian L-PGDS.

A pro-inflammatory role for L-PGDS has been reported in models of allergic inflammation (Satoh et al. 2006) and other inflammation models (Ogawa et al. 2006; Nagata et al. 2009; Uehara et al. 2009; Hokari et al. 2011). Other studies using gain and loss of function strategies in mice have shown an important role for L-PGDS in the innate immune response (Joo et al. 2007; Joo and Sadikot 2012a). Here, we have shown that PGDS1 and PGDS2 expression is induced in different tissues of seabream challenged with V. anguillarum, but with different patterns being the blood tissue where the changes were most evident in both cases. The modulation of the PGDS1 and PGDS2 expression by bacterial challenge was weak compared with the changes observed in the mRNA levels of PTGS2, which lies

upstream in the prostanoid biosynthesis pathway. This means that other PGH_2 derivates could be involved in the clearance of bacteria and the resolution of infection. Together, these results suggest that PGDSs would be the main contributor to host defense against bacterial infection.

Previous studies have shown the involvement of L-PGDS in the production of PGD_2 by macrophages stimulated with LPS or bacterial infection (Joo et al. 2007). However, no reports exist about the contribution of L-PGDS to the production of PGD_2 by neutrophils. Accumulating evidence suggests that seabream AGs could be considered the cell functional equivalent to mammalian neutrophils (Sepulcre et al. 2002; Chaves-Pozo et al. 2004; Chaves-Pozo et al. 2005a; Sepulcre et al. 2007a; Sepulcre et al. 2011a; Cabas et al. 2013). We demonstrate herein that AGs do not express PGDS2 constitutively or after stimulation with PAMPs. However, VaDNA or flagellin stimulation induced PGDS1 expression in AGs in a time-dependent manner. In contrast, the induction of PTGS2 expression in AGs occurs early after stimulation with VaDNA or flagellin. This finding is consistent with the observations made by Joo et al. (2007) that macrophages stimulated with LPS express PTGS2 before L-PGDS expression by differentially using key transcription factors (NF-κB and AP-1, respectively), ensuring that a sufficient amount of PGH_2 is available for L-PGDS to produce PGD_2 (Joo et al. 2007). In agreement with this, the expression of IL-1β, which is also modulated by NF-κB, showed the same kinetics as PTGS2.

L-PGDS is a bifunctional protein, acting as a PGD_2 producing enzyme and also as an intercellular transporter of lipophilic molecules (Fujimori et al. 2006). Previous studies have shown that both zebrafish and chicken PGDSs lack enzymatic activity, while they are able to bind thyroxine and all-trans retinoic acid, as mammalian L-PGDSs and other lipocalin gene family proteins do (Fujimori et al. 2006). However, the role of this amino acid in the activity of fish L-PGDS homologues is not clear, since a zebrafish mutant L-PGDS with reconstituted cysteine (G59C) also lacks enzymatic activity (Fujimori et al. 2006). In view of the observed induction of seabream PGDS1 expression in AGs stimulated with PAMPs, we set about determining the production of PGD_2 by these cells and the contribution of PGDS1 in this process. Our results demonstrate that seabream AGs are able to produce PGD_2 and such production is increased upon stimulation with VaDNA with a similar kinetics to that of PGDS1 expression. These results suggest that PGDS1 may be responsible for the production of PGD_2, which in turn, would be involved in the resolution of the inflammatory response in this species. Although further studies are needed for determine the role of seabream PGDS1 in this process, it is tempting to speculate that it contributes to PGD_2 production by AGs. Very few reports exist regarding the role of PGD_2 production by neutrophils and its possible role in the resolution of inflammation. Taken into account that gilthead seabream AGs are considered the functional equivalent to mammalian neutrophils, these results led us to hypothesize that the production of PGD_2 by neutrophils could be an essential component in the resolution of the inflammation in vertebrates.

The role of PGD_2 in the immune response is complex because it may elicit pro-inflammatory and anti-inflammatory effects, depending on the inflammatory milieu. In addition, PGD_2 can exert its function by interaction with two G protein-coupled receptors: D prostanoid receptor 1 (DP1) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Gervais et al. 2001). CRTH2 has been shown to be expressed in several mouse immune cells, including Th1 cells, Th2 cells, eosinophils, monocytes, and neutrophils (Takeshita et al. 2004; Kim and Luster 2007). In this study, we demonstrate that stable synthetic analogues of PGD_2 (16,16–dimethyl-PGD_2) and its derivate 11-deoxy-11-methylene-15-keto-PGD_2, specific agonist for CRTH2, at micromolar concentrations, decrease the production of ROS by AGs stimulated in vitro with PAMPs. In addition, both of them were able to down-regulate the mRNA levels of IL-1β in stimulated AGs. However, none of the synthetic analogues of PGD_2 tested was able to affect non-stimulated AGs, suggesting that a pro-inflammatory environment favor the anti-inflammatory role of PGD_2. Therefore, these results show that the PGD_2 produced by stimulated AGs exerts an anti-inflammatory effect on its own. Nevertheless, PGD_2 is a relatively unstable molecule, which is readily degraded by a series of spontaneous dehydration and isomerization reactions into biologically active prostaglandins of the J_2 series, namely PGJ_2, $\Delta^{12}PGJ_2$ and 15-deoxy-$\Delta^{12,14}$ PGJ_2, called CyPGs. Therefore, the role of PGD_2 in inflammation is complex because its net effect may depend on the rate of the production of its distal products. In this regards, we demonstrate herein that both CyPGs also exert anti-inflammatory effects on AGs activities by decreasing ROS production and the expression of IL-1β. It is of note that CyPGs were more potent and exerted their effects at lower doses than PGD_2. Thus, it is tempting to speculate that some of the regulatory effects of PGD_2 in the late phases of the resolution of inflammation may also arise from its induction of other families of PGH_2 derivates.

In summary, in this study we provide evidence that PGD_2, probably produced by PGDS1, could play an important role in the resolution of inflammation in gilthead seabream. In addition, we demonstrate that AGs, cells functionally equivalent to mammalian neutrophils, synthesize PGD_2.This molecule directly or via the production of its metabolites CyPGs regulate the resolution of the inflammatory response by controlling the balance of anti- vs. pro-inflammatory mediators, as well as by decreasing ROS production. Furthermore, our results point to the importance of L-PGDS in the production PGD_2 in lower evolutionary advanced vertebrates, which lack H-PGDS.

CHAPTER II: Prostaglandin E_2 promotes M2 polarization of macrophages via a cAMP/CREB signaling pathway and deactivates granulocytes in teleost fish

Abstract

The profile of prostaglandin (PG) production is determined by the differential expression of the enzymes involved in their production and degradation. Although the production of PGE_2 by fish leukocytes has been relatively well studied in several fish species, knowledge of how its production is regulated, its biological activities and the signaling pathways activated by this PG is scant or even contradictory. In this work we show that in the teleost fish gilthead seabream (*Sparus aurata* L.) macrophages regulate PGE_2 release mainly by inducing the expression of the genes encoding for the enzymes responsible for its synthesis, while acidophilic granulocytes (AGs) not only induce these genes quickly after activation but also inhibit the expression of the genes encoding for the enzymes responsible for PGE_2 degradation at later time points. In addition, treatment of macrophages with PGE_2 promoted their M2 polarization, which is characterized by high expression levels of interleukin-10, mannose-receptor c-type 1 and arginase 2 genes. In sharp contrast, PGE_2 promoted the deactivation of AGs, since it decreased the production of reactive oxygen species and the expression of genes encoding pro-inflammatory cytokines. These differences are the result of the alternative signaling pathways used by PGE_2 in macrophages and AGs, a cAMP/CREB signaling pathway operating in macrophages, but not in AGs, downstream of PGE_2. Our data identify for the first time a role for professional phagocyte-derived-PGE_2 in the resolution of inflammation in fish and highlight key differences in the PGE_2 signaling pathway in macrophages and granulocytes.

1. Introduction

Prostaglandins (PGs) comprise a large family of small oxygenated compounds derived from fatty acids that regulate the immune response and inflammation. PGs themselves are derived from the arachidonic acid released from the cell membrane through the action of the enzyme phospholipase A_2 and its subsequent conversion into the intermediate PGH_2 by either of the two isoforms of protaglandin-endoperoxide synthase, the constitutively expressed isoform, PTGS1 (COX1), or the inducible isoform, PTGS2 (COX2) (Simmons et al. 2004a). PGH_2 is converted into PGE_2 through the action of the enzyme PGE synthase (PGES) (Murakami et al. 2002a). Several isoforms of the enzyme PGES have been described in mammals: the cytosolic (cPGES) and the membrane-associated or microsomal (mPGES) PGES (Murakami et al. 2002a). The latter is highly inducible and displays functional coupling with upstream COX-2 enzymes in cells (Murakami et al. 2002a). In addition to the rate of PGE_2 synthesis, the presence of PGE_2 in a specific environment is also regulated by 15-hydroxyprostaglandin dehydrogenase (HPGD)-mediated degradation (Kalinski 2012). The suppression of 15-HPGD activity has been described in PGE_2-rich and immunosuppressive environments (Andrae et al. 2012).

PGE_2 has a dual role modulating multiple aspects of inflammation in a context-dependent manner, showing proinflammatory and anti-inflammatory activities (Kalinski 2012). In mammals, PGE_2 has been described as regulating the function of several immune cells, particularly those involved in innate immunity such as macrophages, neutrophils or natural killer (Kalinski et al. 1997; Hoshino et al. 2007; Kalinski 2012). Macrophages are key components in regulation of the immune response as well as in inflammation. Depending on the set of specific signals in the vicinity of the cells, they take on distinct phenotypes to undertake different functions in a process known as macrophage polarization (Mosser 2003; Mosser and Edwards 2008; Sica and Mantovani 2012). Macrophages are an important source of PGE_2 which acts in an autocrine or paracrine fashion (Harris et al. 2002a). For example, PGE_2 inhibits the bactericidal properties of macrophages, including phagocytosis (Aronoff et al. 2004; Kalinski 2012) or the production of reactive oxygen species (ROS) (Serezani et al. 2007). It also intercedes in the balance and type of immunoregulatory cytokines such as IL-10 or IL-17 (Kunkel et al. 1988; Hoshino et al. 2007; Hirata and Narumiya 2012a), influencing, among other things, the macrophage phenotype (Mosser and Edwards 2008; Ricciotti and FitzGerald 2011; MacKenzie et al. 2013; Rodriguez et al. 2014). Neutrophils are important cell effectors of the innate immune response, whose main functions are phagocytosis and the release of ROS, proteolytic enzymes and inflammatory mediators. PGE_2 has been seen to be generated by neutrophils following treatment with pathogens or their structural components (Herrmann et al. 1990; Pouliot et al. 1998; He et al. 2001; Alba-Loureiro et al. 2004; St-Onge et al. 2007). In a similar way to macrophages, PGE_2 exerts inhibitory effects on neutrophil functions such as superoxide anion production (Sedgwick et al. 1985; Gryglewski et al. 1987; Hecker et al. 1990b; Wheeldon and Vardey 1993), chemotaxis (Armstrong 1995), the release of cytotoxic enzymes (Hecker et al. 1990a) and aggregation (Wise 1996).

The variety of effects that PGE_2 can elicit reflects the presence of specific PG receptors in many cells types. PGE_2 signals through four distinct G protein-coupled E prostanoid (EP) receptors (EP1-EP4), which differ in their affinity and signaling duration (Hoshino et al. 2007; Hirata and Narumiya 2011b; Kalinski 2012; Alasoo et al. 2015; Cobos Jimenez et al. 2015). These receptors couple to a range of intracellular signaling pathways that mediate the effects of receptor activation on cell function. EP2 and EP4 receptors activate adenylyl cyclase, increasing cAMP (Ricciotti and FitzGerald 2011). EP1 activates the phophatidylinositol metabolism, leading to the formation of inositol triphosphate and the mobilization of intracellular free calcium, while the EP3 isoform leads to higher intracellular calcium levels and the inhibition of cAMP (Ricciotti and FitzGerald 2011).

Several studies have already demonstrated the production of PGE_2 by leukocytes, mainly macrophages, in different fish species, as well as its effects on immune-relevant activities. Most studies have been performed using total leukocytes from head kidney (the equivalent to mammalian bone marrow). In this context, the exogenous addition of PGE_2 to trout head kidney leukocytes has been shown to inhibit their proliferative response (Secombes et al. 1994b), to up-regulate the expression of IL-10, IL-6 and COX-2, and down-regulate IFNγ , TNFα (Belmonte et al. 2014a) and MHCII expression (Secombes et al. 2001c). In addition, PGE_2 has been shown to modulate ROS production in head kidney leukocytes from sticklebacks (Kutyrev et al. 2014a) and turbot (Parama et al. 2007). The very few studies conducted in head kidney macrophages have been performed in trout, where PGE_2 potentiates the phagocytic response (Knight et al. 1993a) and inhibits ROS production (Novoa et al. 1996b). However, the expression profiles of the enzymes that regulate PGE_2 production in fish phagocytes, its biological activities in different phagocyte populations and the intracellular signaling pathways that mediate its effects are largely unknown.

The aim of the current study was to gain further insight into the role of PGs in the resolution of inflammation in gilthead seabream (*Sparus aurata* L.), an immunological tractable teleost model. The results point to a high degree of regulation of the genes encoding for the enzymes involved in PG production and degradation *in vivo* after bacterial challenge, as well as *in vitro* in macrophages and AGs stimulated with PAMPs. Functional studies also showed that PGE_2 promotes M2 and anti-inflammatory phenotypes in macrophages and AGs, respectively, possibly reflecting the differential signaling pathways used by PGE_2 in each phagocytic cell type.

2. Materials and Methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinoperygii, Sparidae) were bred and kept at the Oceanographic Centre of Murcia (Spain) in a 14 m^3 running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Seabream were terminated via cervical dislocations using approved procedures following anaesthetization with clove oil. All efforts were made to minimize animal stress and to ensure that termination procedures were performed efficiently.

2.2. In vivo sampling and experimental infections

Fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10^8) of exponentially growing *Vibrio anguillarum* R82 cells (Chaves-Pozo et al. 2004). Head kidney, spleen, thymus, liver, gills, blood and peritoneal exudates cells were obtained 4 h after bacterial challenge since strong acidophilic granulocytes recruitment and activation was observed at this time point (Chaves-Pozo et al. 2004). All samples were processed for subsequent real-time RT-PCR (see below).

2.3. Isolation of phagocytes

AGs were obtained by MACS as described earlier (Roca et al. 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) (Sepulcre et al. 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100-200 μl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al. 2006). Head kidney macrophage monolayers were then obtained after overnight culture of $G7^-$ fractions in serum-free medium as described earlier (Roca et al. 2006).

2.4. Cell culture and treatments

Phagocytes were stimulated at 23°C with 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) (Pelegrin et al. 2004) or 100 ng/ml flagellin (Invivogen) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33

mOs) with 0.35% NaCl] supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 µg/ml strepomycin (Biochrom). These concentrations of PAMPs have been found to be optimal for the *in vitro* activation of seabream AGs (Sepulcre et al. 2007a; Sepulcre et al. 2011b). AGs and macrophages were cultured with or without PGE_2 (1-10 µM), or the synthetic stable analog of PGE_2 (1-10 µM, 16,16-dimethyl PGE_2) (Cayman Chemical). In some experiments, purified AGs macrophages were incubated with 0.2 mM cell-permeable cAMP analog 29-dibutyryladenosine 39,59-cyclic monophosphate sodium salt (dbcAMP; Sigma-Aldrich) for 6 h and 30 min, respectively (Cabas et al. 2013).

2.5. Analysis of gene expression

Total RNA was extracted as indicated above and treated with Amplification grade DNase I (1 unit/µg RNA, Life technologies). SuperScrip III RNase H^- ReverseTranscripase (Life technologies) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1 µg of total RNA at 50ºC for 50 min. Real-time PCR was performed with an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the ribosomal protein S18 (*rps18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ for the constitutive expression analysis). The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice.

Gene	**Accession number**	**Name**	**Sequence (5' -3')**
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
il10	FG261948	F	TGGAGGGCTTTCCTGTCAGA
		R	TGCTTCGTAGAAGTCTCGGATGT
mrc1	FG264028.1	F	GTTTTGACAGTTGGGCTGGT
		R	CCTGAATCGCTCATTGTTGA
arg1	HS988184	F2	AGAGGCGGTTAAACTGGGTG
		R2	TCGATGTCGAAGCTCAGGTG
arg2	FM144483	F2	TCTCTAGCGGTGGACGTCAT
		R2	CTAGAGGCGGAGTTGCTCTG
ptgs2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
hpgd		F1	CGGCAAGGAAGGAGGCACCA
		R1	CGGACGCCGTAGTCACCCTG
pges	FM153443	F	GCTGAGACACGGAGGGTTAC
		R	GTCCAGTCAAGGCGTACACA
rps18	AY587263	F1	AGGGTGTTGGCAGACGTTAC
		R1	CTTCTGCCTGTTGAGGAACC

Table 1. Gene accession numbers, and primer sequences used for gene expression analysis

2.6. Cell viability

Aliquots of cell suspensions were diluted in 200 μl PBS containing 40 μg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by flow cytometry.

2.7. Respiratory burst assays

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by AGs after different stimulation times (Mulero et al. 2001). This was done by adding 100 μM luminol and 1 μg/ml PMA (both from Sigma-Aldrich), while the chemiluminescence was recorded every 117 seconds for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the

average of quadruple readings, expressed as the slope of the reaction curve from 117 to 1170 seconds, from which the apparatus background was subtracted.

2.8. Western blot

Macrophages untreated or treated with PGE_2 or 0.2 mM dbcAMP for 3 h, as described above, were lysed in lysis buffer (10 mM Tris-HCl [pH 7.4], 150mM NaCl, 1% Triton X-100, and 0.5% Nonidet P40). The protein concentrations of cell lysates were estimated with the bicinchoninic acid protein assay reagent (Pierce) using BSA as a standard. Cell extracts (10 or 45 mg of protein) were boiled in SDS sample buffer, resolved on 12% SDS-PAGE, and transferred for 30 min at 200 mA to nitrocellulose membranes (Bio-Rad). Blots were probed with 1:1000 dilution of a commercial rabbit mAb raised against cAMP response element-binding protein (CREB) phosphorylated at serine 133 (phospho-CREB, which also reacts with phospho–activating transcription factor-1) (9198; Cell Signaling Technology) (Cabas et al. 2013). Then blots were probed with 1:5000 of an anti-rabbit HRP Aband developed with ECL reagents (GE Healthcare), according to the manufacturer's protocol. Membranes were then reprobed with a 1:5000 dilution of an affinity-purified rabbit polyclonal to histone H3 (ab 1791; Abcam). The intensity of the bands was estimated using Image J software and semi-quantitative analysis for each sample was performed relative to the histone H3 expression according to the formula: intensity of target band/intensity of its corresponding H3 band.

2.9. Statistical analysis

Data were analyzed by ANOVA and a Tukey's multiple range test to determine differences between groups.

3. Results

3.1. In vivo expression of gilthead seabream PGES and modulation upon bacterial infection

The PGE_2 content in a specific inflammatory milieu depends on the balance of its synthesis *vs* its degradation. To obtain further insight into the regulation of PGE_2 production in gilthead seabream after *in vivo* bacterial challenge, RT-qPCR was used to analyze the expression of gene encoding for the enzymes involved in its synthesis and degradation, namely Pges and Hpgd. Seabream *pges* was expressed in all the tissues examined, including the key immune organs, head kidney, spleen, thymus and gills (Figure 1A). The highest mRNA levels of *pges* were found in gill and spleen, while the lowest in blood and peritoneal exudate (Figure 1A). Infection of fish with the pathogenic bacterium *V. anguillarum* increased the mRNA levels of *pges* in the key immune organs analyzed, namely thymus, spleen, head kidney and peritoneal exudate (the site of bacterial injection) (Figure 1B). By contrast, the mRNA levels of *pges* did not significantly change in liver and gill, and even decreased in blood, after

bacterial challenge. However, the expression of *hpgd* was hardly altered in the tissue tested with the exception of liver and head kidney, which showed decreased expression (Figure 1C). These results suggest a role for PGE_2 in the early phases of the resolution of the inflammatory response in gilthead seabream, the amount involved being controlled mainly by its synthesis rather than its degradation.

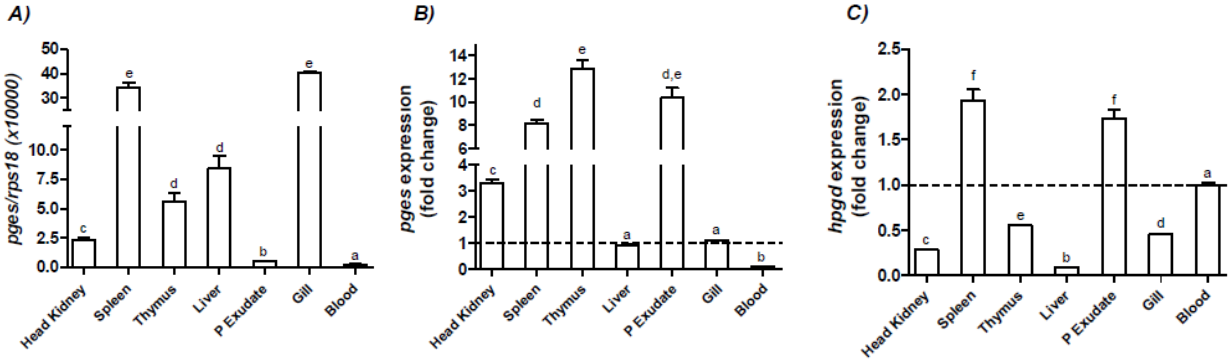

Figure 1. In vivo expression of genes encoding for the enzymes involved in PGE_2 synthesis and degradation, constitutively and following infection. The mRNA levels of *pges* (A, B) and *hpgd* (C) were determined by real-time RT-PCR in the indicated immune tissues of control specimens or following a 4 h challenge with 10^8 cells of *Vibrio anguillarum*. The constitutive expression is showed relative to *rps18* and the regulation of the expression following infection is shown as the mean ± S.E of the mRNA fold change in infected fish relative to control fish (indicated with horizontal dashed lines) (B, C). Different letters denote statistically significant differences among the groups according to a Tukey test ($p<0.05$). The groups marked "a" did not show statistically significant differences from non-infected fish.

3.2. Stimulation of gilthead seabream professional phagocytes with PAMPs resulted in different expression profiles of the enzymes involved in PGE2 production

We next analyzed the expression of the genes coding for the main enzymes involved in PG biosynthesis in seabream macrophages and AGs activated *in vitro* with genomic DNA from *V. anguillarum* (VaDNA). The results show that stimulation of macrophages with VaDNA resulted in increased expression of the gene encoding for the limiting enzyme in PG production, Ptgs2, throughout the stimulation-time, peaking at 3 h (Figure 2A). Interestingly, the mRNA levels of *pges* increased with the same kinetics as that of *ptgs2* (Figure 2B), while those of *hpgd* were hardly induced in macrophages stimulated with VaDNA (Figure 2C). All these results further suggest that PGE_2 levels are controlled by the induction of the genes encoding for the enzymes responsible of its biosynthesis.

Regarding AGs, stimulation of these cells with VaDNA or flagellin resulted in dramatically increased transcript levels of *pges* at the earliest time point analyzed (24 h) (Figure 3A). In addition, the mRNA levels of *hpgd* gradually declined in AGs, reaching their lowest levels at 3 and 10 d for VaDNA and flagellin, respectively (Figure 3B). These results point to AG-derived-PGE_2 as a key component in

the regulation of the early phases of inflammation, the exact amount being regulated by the early induction of the gene encoding for the enzyme responsible for its synthesis and the subsequent down-regulation of the gene encoding for the enzyme responsible for its degradation.

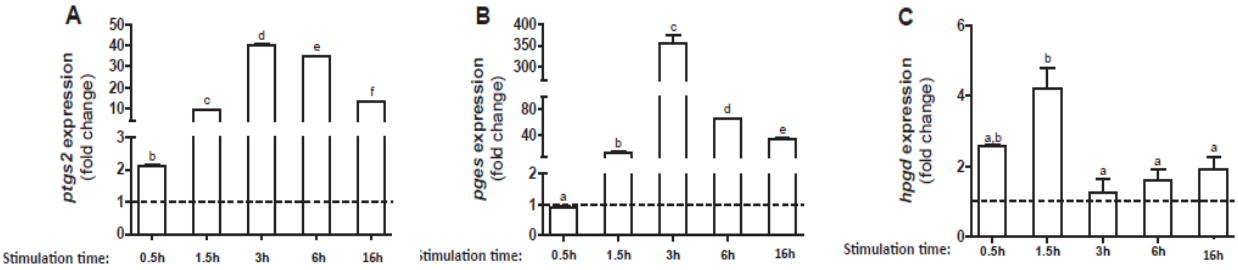

Figure 2. Modulation of the expression profile of genes encoding for the enzymes involved in PG synthesis and degradation in macrophages upon stimulation. Macrophages from head kidney were stimulated for 0.5, 1.5, 3, 6 and 16 hours with 50 µg/ml VaDNA in sRPMI supplemented with 0.15% FCS, and the mRNA levels of *pgts2* (A), *pges* (B) and *hgpd* (C) were studied by real-time RT-PCR. The gene expression is normalized against *rps18* and expressed as mean±S.E. of the gene mRNA fold increase in stimulated cells relative to non-stimulated cells (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p<0.05$). The groups marked "a" did not show statistically significant differences from control cells.

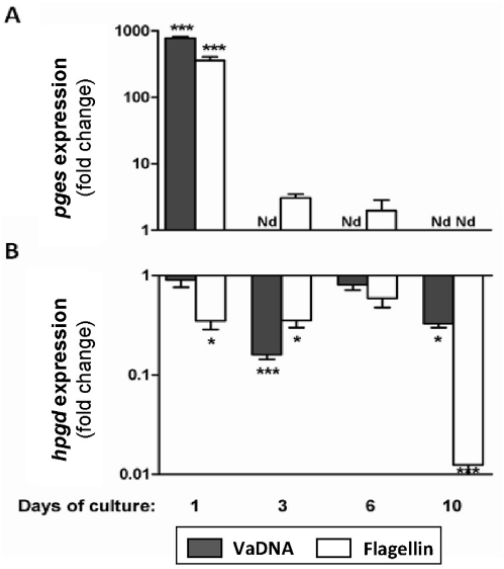

Figure 3. Modulation of the expression profile of genes encoding for the enzymes involved in PGE_2 synthesis and degradation in AGs upon stimulation. AGs from head kidney were stimulated for 1, 3, 6 and 10 days with 50 µg/ml VaDNA or 0.1µg/ml flagellin in sRPMI supplemented with 5% FCS and the mRNA levels of *pges* (A) and *hgpd* (B) were studied by real-time RT-PCR .The gene expression is normalized against *rps18* and expressed as mean±S.E. of the gene mRNA fold change in stimulated cells relative to non cultured cells. Statistically significant differences from non-infected fish according to a Tukey test are shown as: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.3. PGE2 promotes the M2 polarization of gilthead seabream macrophages

To determine the biological activity of PGE_2 in gilthead seabream macrophages, head kidney macrophages were treated with PGE_2 for 6 h in the presence or absence of VaDNA and the expression of key immune-related genes was analyzed by RT-qPCR. The results show that PGE_2 increased the expression of *il10* (Figure 4A) but not *il1b* transcript levels (Figure 4B) and inhibited those of *il6* in macrophages co-stimulated with VaDNA. However, it had negligible effects, if any, in non-stimulated cells. Considering that the production of IL-10 is a key characteristic of M2 macrophages, we sought to determine the expression of some genes typical of the M2 phenotype, such as *mrc1* and *arg*. PGE_2 increased the mRNA levels of both *mrc1* (Figure 4C) and *arg2* (Figure 4D) in control and VaDNA-stimulated macrophages, although the highest induction was observed in the stimulated macrophages. However, PGE_2 did not affect the mRNA levels of *arg1* (Figure 4E) in control or in VaDNA-stimulated macrophages.

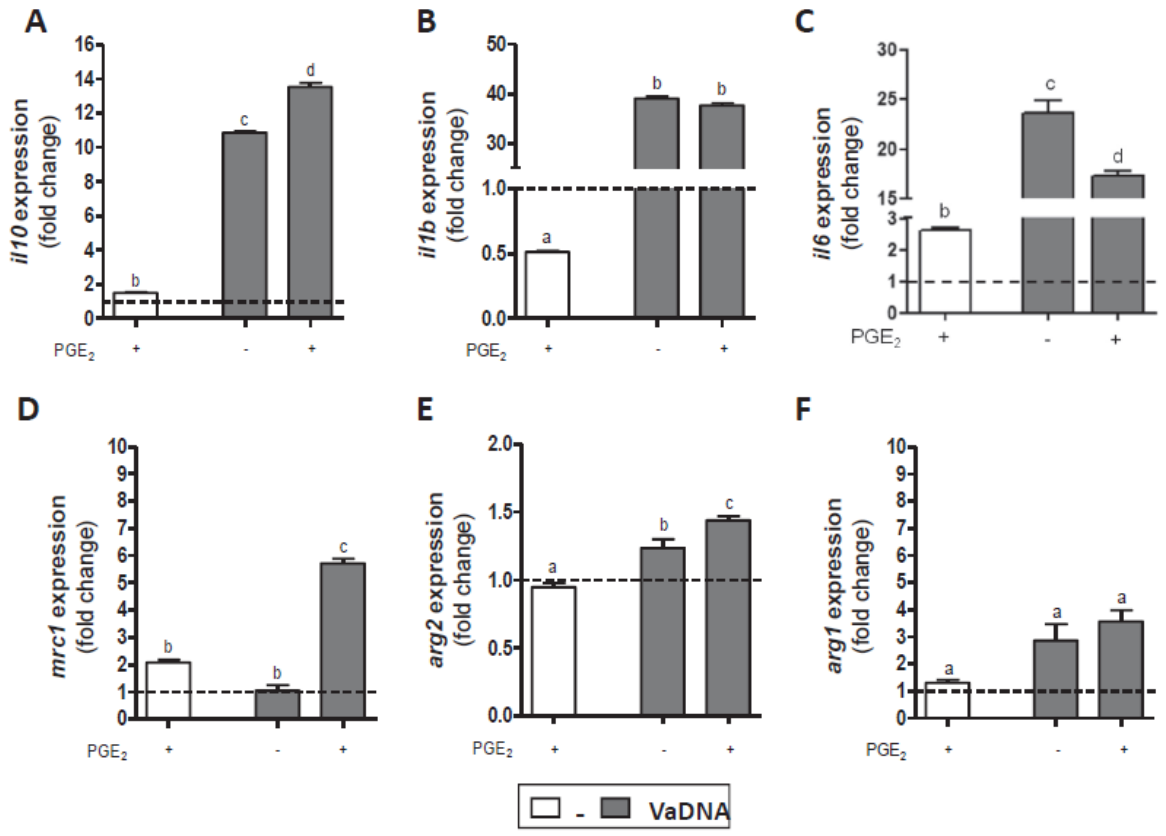

Figure 4. PGE_2 promotes the M2 polarization of gilthead seabream macrophages. Head kidney macrophages were incubated for 6 h with PGE_2 (10 μM), with or without 50 μg/ml of *Va*DNA, and the mRNA levels of the gene coding for IL-10 (A), IL-1β (B), IL-6 (C), MRC1 (D) and arginase2 (E) and arginase 1 (F) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and is expressed as mean±S.E. of mRNA fold change in stimulated cells relative to control cells incubated in medium alone (indicated with horizontal dashed

lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p<0.05$). The groups marked "a" did not show statistically significant differences from control cells.

3.4. PGE2 inhibits gilthead seabream AG functions

We next determined the biological participation of PGE_2 in major gilthead seabream AG functions, such as the production of ROS and the expression of genes encoding for major cytokines. Taking into account the low stability of PGs, the half life of PGE_2 of 30 s in the circulatory system (Fitzpatrick et al. 1980b) and 26 h in culture medium (Watzer et al. 2009a), and the delayed response of AGs in the induction of genes encoding for pro-inflammatory cytokines after PAMP stimulation compared with macrophages (Sepulcre et al. 2007a), head kidney AGs were treated with PGE_2 or 16,16-dimethyl-PGE_2, a metabolically stable synthetic analog of PGE_2, in the presence or absence of VaDNA. The results showed that both PGs inhibited the production of ROS primed by VaDNA at all the times tested (i.e. 6, 16 and 36 h) (Figures 5A-C), while cell viability was unaffected (data not shown). According to these results, both PGE_2 and 16,16-dimethyl-PGE_2 were able to decrease the mRNA levels of *il1b* (Figures 5D and 5H) and *il10* (Figures 5E and 5I) and *il6* (Figures 5F and 5J) induced by VaDNA at the longest time tested (16 h), while 16,16-dimethyl-PGE_2 was also able to significantly decline *il6* transcript levels at 6h (Figure 5F).

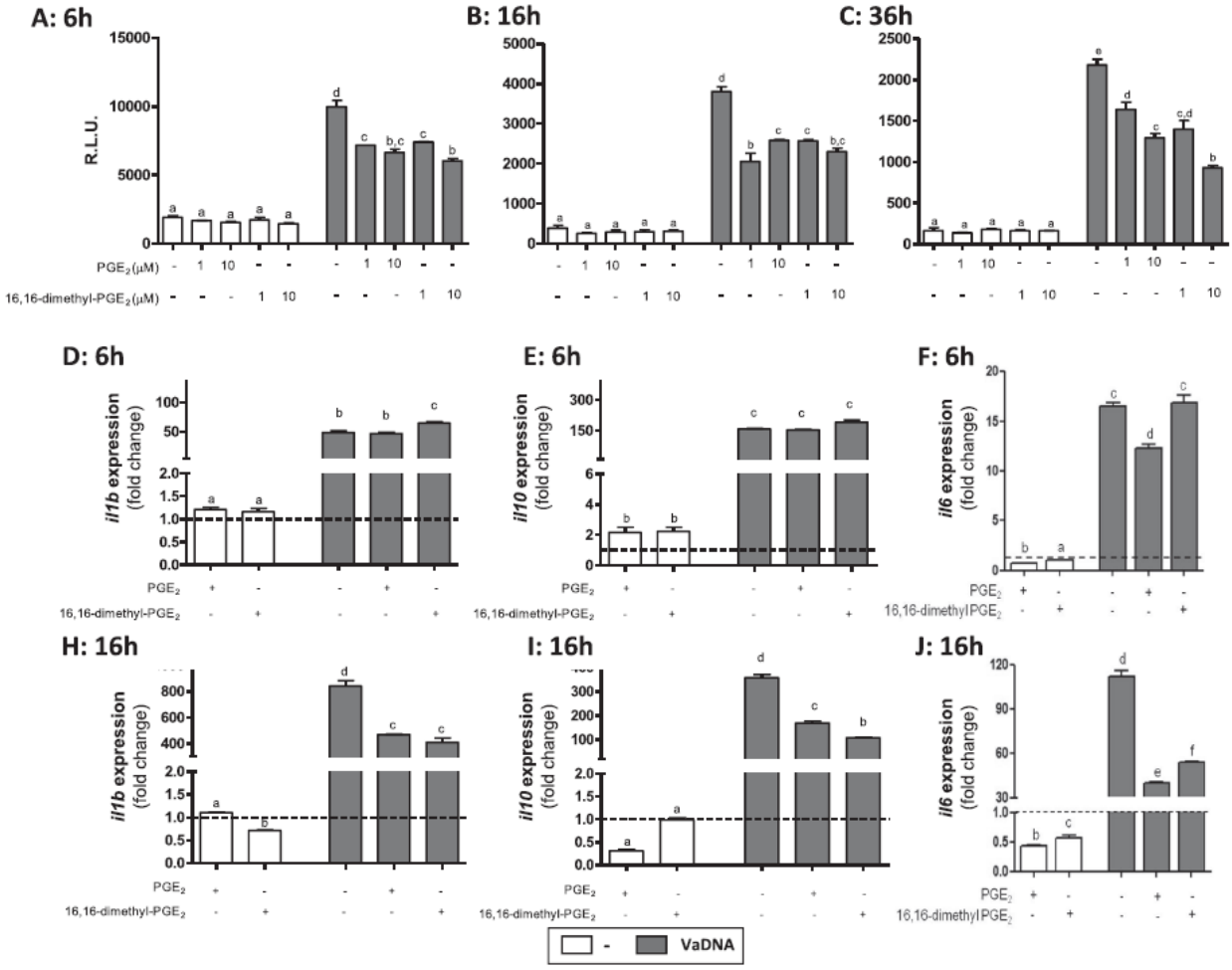

Figure 5. PGE_2 inhibits gilthead seabream AG functions. Purified AGs from head kidney were incubated for 6 h (A,D,E, F), 16 h (B,H, I, J) and 36 h (C) with the indicated concentrations of PGE_2 or 16,16–dimethyl-PGE, with or without 50 μg/ml of VaDNA, and their respiratory burst activity was measured (A-C). The mRNA levels of the gene coding for IL-1β (D, H), IL-10 (E, I) and IL-6 (F, J) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and is shown as the mean±S.E. of the mRNA fold change relative to the mean of control cells incubated in medium alone (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p<0.05$). The groups marked "a" did not show statistically significant differences from control cells.

3.5. PGE2 signals through a cAMP/PKA/CREB pathway in macrophages

In mammals, of the four PGE_2 receptors, EP2 and EP4 are the most relevant for the inflammatory responses. Their downstream signaling leads to increased cAMP concentrations, activating, in turn, the PKA/CREB pathway (Ricciotti and FitzGerald 2011). The non-availability of gilthead seabream PGE_2 receptor sequences and that the ability of cAMP to act as an alternative activator of fish macrophages (Joerink et al. 2006a) led us to explore the signaling pathway activated by PGE_2 in

macrophages and AGs by using a cell-permeable analog of cAMP that activates PKA (dbcAMP). The results showed that dbcAMP mimicked the effect of PGE_2 on the regulation of *il1b* and *il10* transcript levels in VaDNA-stimulated macrophages (Figures 6A and 6B) but not in AGs (Figures 7A and 7B). Consistent with these results, PGE_2 promoted CREB and ATF-1 phosphorylation in macrophages stimulated with VaDNA (Figure 6C). All these results suggest that PGE_2 signals through EP2 and/or EP4 in seabream macrophages but not in AGs and highlight the diversity of functions of these two cell types. Further studies are needed to confirm the presence of PGE_2 receptors and signaling in fish professional phagocytes.

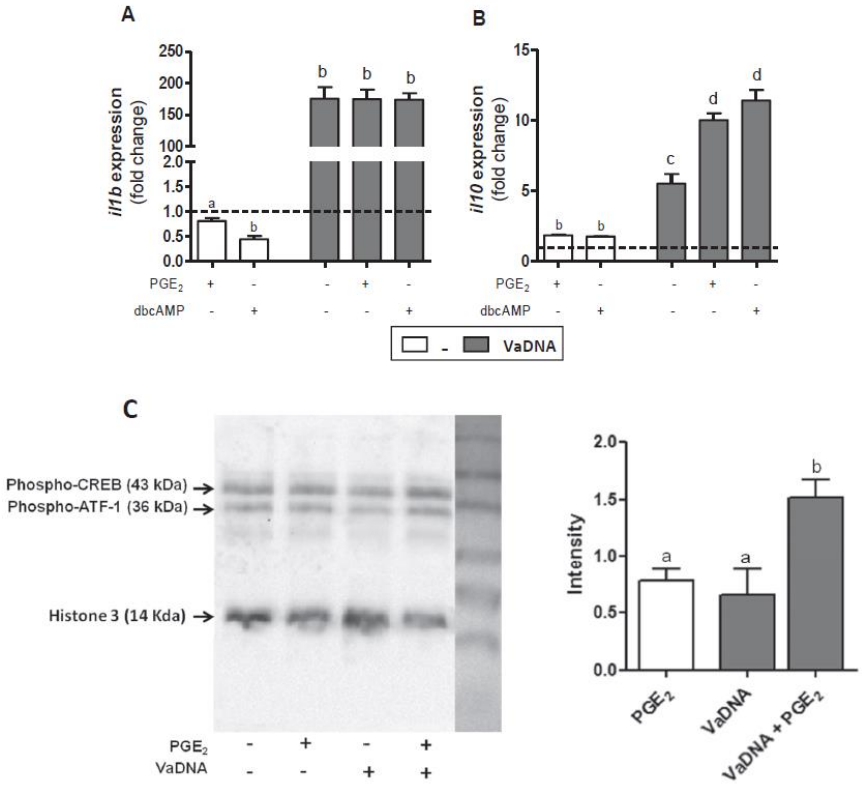

Figure 6. PGE_2 signals through a cAMP/PKA/CREB pathway in macrophages. (A, B) Macrophages from head kidney were incubated for 3 h with PGE_2 (10 μM) with or without 50 μg/ml of VaDNA or with 0.2 mM dbcAMP for 30 min. Afterwards, the mRNA levels of the gene coding for IL-1β (A) and IL-10 (B) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and is shown as the mean±S.E. of the mRNA fold change relative to the mean of control cells incubated in medium alone (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p<0.05$). The groups marked "a" did not show statistically significant differences from control cells. (C) Western blot analysis of Phospho-CREB (Ser133) and histone H3 in AGs untreated or treated with 10 μM PGE_2 with or without 50 μg/ml of VaDNA or with 0.2 mM dbcAMP for 30 min. The intensity of the bands was estimated using Image J software and semi-quantitative analysis for each sample was performed relative to the histone H3 expression according to the formula: intensity of target band/intensity of its corresponding H3 band. The results are shown as the mean ± S.E of two independent experiments performed with pools of macrophages from 3 specimens.

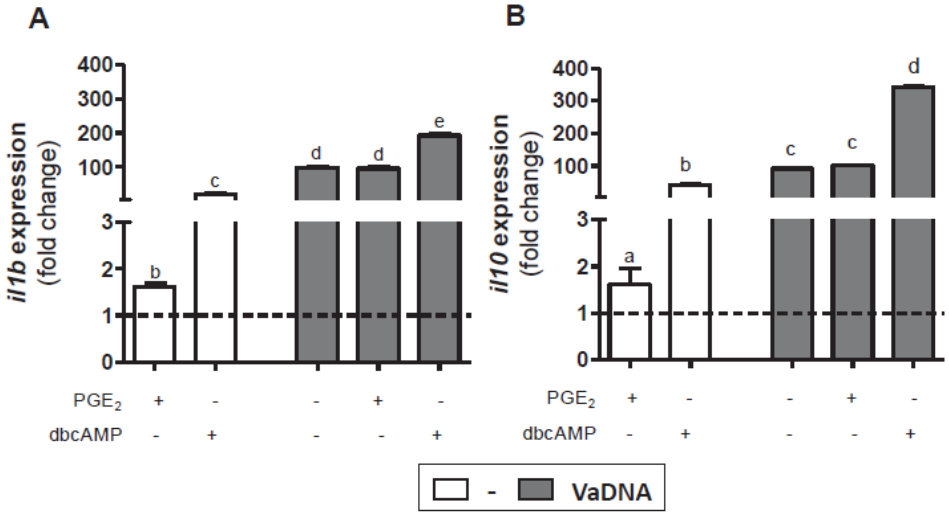

Figure 7. Cyclic AMP does not mimic the PGE_2 effect in seabream AGs. AGs purified from head kidney were incubated for 6 h with PGE_2 (10 μM) or with 0.2 mM dbcAMP, with or without 50 μg/ml of *Va*DNA. The mRNA levels of the gene coding for IL-1β (A), IL-10 (B) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and is shown as the mean±S.E. of the mRNA fold change relative to the mean of control cells incubated in medium alone (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test. The groups marked "a" did not show statistically significant differences from control cells ($p<0.05$).

4. Discussion

The present study, throws light on some important aspects of PGE_2 biosynthesis in the teleost model gilthead seabream. The first of these was the regulation of the PGE_2 content in major immune relevant organs in fish experimentally infected with the fish pathogenic bacterium *V. anguillarum.* The second was the diversity of mechanisms operating in professional phagocytes in the regulation of PG production. The rate of PG synthesis is primarily due to the expression and activity of the immune-related inducible enzyme PTGS2 (Kalinski 2012) . To date, many studies have demonstrated the presence of mammalian homologs of *ptgs2* in a variety of fish species and their modulation under stimulation; for example in zebrafish (*Danio rerio*) (Grosser et al. 2002b), rainbow trout (*Oncorhynchus mykiss*) (Boltana et al. 2011; Belmonte et al. 2014a), gilthead seabream (*Sparus aurata*, L.) (Sepulcre et al. 2007a; Mulero et al. 2008b; Sepulcre et al. 2011a; Lopez-Munoz et al. 2012; Boltana et al. 2014a; Gomez-Abellan et al. 2015), European seabass *(Dicentrarchus labrax*) (Sepulcre et al. 2007b), Atlantic cod (*Gadus morhua*) (Holen et al. 2012b) and Atlantic salmon (Sal*mo salar*) (Fast et al. 2005b). The data presented herein demonstrate the concomitant up-regulation of the mRNA levels of both *ptgs2* (Sepulcre et al. 2007a) and *pges*, the most limiting enzyme in PGE_2 synthesis, in gilthead seabream macrophages following stimulation with VaDNA. This data support the observations made by Boltaña

et al. who described a correlation of *ptgs2* expression and the production of PGE_2 by gilthead seabream macrophages stimulated with different PAMPs (Boltana et al. 2014a). In mammals, there are several isoforms of PGES, namely cPGES and mPGES (Murakami et al. 2002a), the latter being a highly inducible isoform that displays functional coupling with upstream PTGS2 in phagocytes (Murakami et al. 2002a). The existence of mPges1 and cPges homologs has been reported in zebrafish, along with an apparent correlation between mPges1 and Ptgs2 expression profiles (Pini et al. 2005b). In view of the expression pattern of gilthead seabream Pges, it is tempting to consider it as functionally equivalent to mammalian mPGES.

In addition to the rate of PGE_2 biosynthesis, the levels of PGE_2 in a specific environment are also regulated by its degradation via HPGD (Kalinski 2012). Interestingly, no modulation of the expression of *hpgd* was observed in VaDNA-stimulated gilthead seabream macrophages, suggesting a pivotal role for Ptgs2 and Pges in the regulation of the amount of PGE_2 available during the inflammatory process. All these results suggest a role for macrophage-derived PGE_2 in the regulation of the early phases of the inflammatory process, while PGD_2 will operate at later stages (Boltana et al. 2011; Gomez-Abellan et al. 2015).

As regards AGs, which are functionally equivalent to mammalian neutrophils (Sepulcre et al. 2002; Chaves-Pozo et al. 2004; Sepulcre et al. 2007a; Sepulcre et al. 2011a; Cabas et al. 2013), the present study points to a different scenario for the regulation of PGE_2 biosynthesis. Stimulation of AGs with different PAMPs resulted in a dramatic increase in the mRNA levels of *pges* at the earliest time point analyzed (24 h), while the expression of *hpgd* was gradually down-regulated with longer stimulation time. Therefore, the levels of AG-derived PGE_2 are fine-tuned by the early up-regulation of the gene encoding for the enzyme responsible for its synthesis and subsequent down-regulation of the gene encoding for the enzyme responsible for its degradation. It is also important to note that, in contrast to macrophages, the expression of *pges* in AGs did not show the same kinetics as *ptgs2*, which remained up-regulated after 3 days of stimulation (Gomez-Abellan et al. 2015). This result is in agreement with a previous study showing that in AG-derived PGD_2 also plays an important role in the resolution of inflammation (Gomez-Abellan et al. 2015). Taken together, these results point to a key role for both macrophages and AGs in the regulation of inflammation in gilthead seabream through the production of PGE_2.

The microenvironment exerts a profound effect on macrophages, generating states of activation that differ in phenotype and function (Munder et al. 1999; Stout et al. 2009). In recent years, a variety of terms have been applied to refer to this heterogeneity (Murray et al. 2014). In parallel with the Th1/Th2 dichotomy, two well-established polarized phenotypes are often referred to as classically activated macrophages (M1) and alternatively activated macrophages (M2) (Mosser 2003; Gordon and Taylor 2005; Rodriguez et al. 2014). M1 macrophages are associated with a pro-inflammatory

phenotype, while M2 macrophages are associated with anti-inflammatory and homeostatic functions (Murray et al. 2014). M2-associated markers include arginase, IL-10 and MRC1 (Lawrence and Natoli 2011). In addition, this classification of M1/M2 macrophages has expanded to account for different activation scenarios (Mantovani et al. 2004; Mosser and Edwards 2008; Murray et al. 2014). Forlenza and coworkers reviewed the four phenotypes for fish macrophages based on the stimulus and the response unleashed: innate, classical-activated, alternative-activated or regulatory macrophages (Forlenza et al. 2011). Although it has been proposed that the term "regulatory" macrophages should be avoided, because all macrophages are regulatory in some capacity (Murray et al. 2014), this phenotype would be generated by PAMPs associated with a second signal, such as immune complexes or PG, that would result in high levels of IL-10. In agreement with studies performed on mammalian macrophages (Strassmann et al. 1994; MacKenzie et al. 2013), the present study shows that PGE_2 increases the mRNA levels of *il10*, *mrc1* and *arg2* and decreased those of *il6* in gilthead seabream macrophages, especially when combined with VaDNA, pointing to the ability of PGE_2 to switch macrophage phenotype in fish, too. Previous studies conducted with carp macrophages have shown a correlation between *il10* and *arg2* expression and alternative-activated macrophages (Strassmann et al. 1994; Forlenza et al. 2011), and our results further confirm the use of these genes as potential markers of M2 phenotype in teleost fish and *mrc1* as an additional M2 marker. Notably, although the addition of PGE_2 concomitantly with VaDNA increased the mRNA levels of *il10*, *arg2* and *mrc1*, and decreased those of *il6*, high levels of *il1b* mRNA were also found. This suggests that PGE_2 promotes an intermediate phenotype between alternatively and regulatory activation, as Forlenza and coworkers described (Forlenza et al. 2011), similar to the M2b phenotype described by Mantovani and coworkers (Mantovani et al. 2004).

Although it has also been suggested that neutrophils polarize towards N1 and N2 phenotypes in similar way to macrophages (Mantovani et al. 2011), to the best of our knowledge, there is no evidence concerning the involvement of PGs in neutrophil polarization. We have previously shown that sustained stimulation of AGs with different PAMPs increases their functional pro-inflammatory capacity, resulting in a N1 polarized phenotype (Sepulcre et al. 2011a). The present study, together with previous results on PGD_2 (Sepulcre et al. 2011a), shows that both PGE_2 and PGD_2 in combination with PAMPs decreased *il1b* and *il6* expression and the production of ROS in AGs. Therefore, it seems reasonable to speculate that PGs are able to deactivate fish granulocytes but are not involved in their polarization. Curiously, our results also show that PGE_2 decreased the expression of *il10* elicited by VaDNA in AGs. The expression of this cytokine in mammalian neutrophils remains controversial. While an induction of IL-10 during infections has been observed in mouse (Mantovani et al. 2011), human neutrophils cannot switch on the IL-10 gene because its locus is in an inactive state (Davey et al. 2011). In fish, although several studies have shown the induction of *il10* under different pathological conditions (Savan et al. 2003; Inoue et al. 2005; Zhang et al. 2005; Belmonte et al. 2014a), no studies have been conducted in fish neutrophils. Taking into account that seabream AGs are considered functionally equivalent to

mammalian neutrophils, this is the first report to demonstrate the modulation of *il10* by PAMPs in fish neutrophils and its fine-tuning by PGE_2.

PGE_2 acts through binding of one or more of its receptors, termed EP1-EP4. Previous studies conducted with mouse macrophages have shown that PGE_2 induces IL-10 via the cAMP/PKA/CREB pathway (Strassmann et al. 1994; MacKenzie et al. 2013). The transcriptional regulation of IL-10 in macrophages is complex, and a role for CREB has been proposed (Saraiva and O'Garra 2010). In addition, several studies have shown that agents that increase cAMP levels, including PGs, can enhance IL-10 transcription (Strassmann et al. 1994; Eigler et al. 1998; Alvarez et al. 2009; Kim et al. 2011), Furthermore, treatment of carp macrophages with cAMP has been associated with a regulatory M2 phenotype (Joerink et al. 2006a; Joerink et al. 2006b). The present results show that while cAMP mimics the effect of PGE_2 in gilthead seabream macrophages activated by PAMPs, it fails to do so in AGs. Supporting this functional study, PGE_2 and PAMP stimulation increased the phosphorylation of CREB in macrophages but not in AGs. Of the four EP receptors, only EP2 and EP4 signal through cAMP in mammals (Ricciotti and FitzGerald 2011), so it is tempting to speculate that PGE_2 acts via EP2 or EP4 in gilthead seabream macrophages but not in AGs. However, further studies are needed to look into the presence EP receptors and the molecular mechanism by which PGE_2 exert its effect in fish professional phagocytes.

In summary, we have shown for the first time that the switch of macrophages to an M2 by the combination of PAMP and PGE_2 is a conserved feature of macrophages from fish to mammals. Furthermore, PGE_2 also regulates the main activities of AGs in a similar way to that which occurs in mammalian neutrophils. These results support the usefulness of fish models for understanding the evolution of phagocyte biology.

CHAPTER III: A role for PPARβ in the anti-inflammatory effect of the cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-PGJ_2 in professional phagocytes of the teleost gilthead seabream

Abstract

Prostaglandins are small lipophilic molecules with a high reactivity derived from cell membrane polyunsaturated fatty acid. 15dPGJ$_2$ is a cyclopentenone PG (cyPG) of the J series with anti-inflammatory, anti-proliferative and pro-apoptotic effects. This cyPG can signal through DP2 receptor, PPARγ receptor or through covalent binding to protein nucleophiles, such as, thiols groups of cysteine, lysine or histidine through a Michael addition reaction modifying its structure and function. In this work we show that in the teleost fish gilthead seabream (*Sparus aurata* L.) acidophilic granulocytes, PPARα, PPARβ and PPARγ are constitutively expressed showing the highest expression for PPARγ and up-regulation upon VaDNA stimulation. In addition, 15dPGJ$_2$ was able to decrease ROS production, as well as, to downregulate the transcription levels of *il1b*, *ptgs2* and *pparg*. By contrast, its biotinylated analog did not elicit the same effects, neither on ROS production nor on cytokine expression. However, CAY10410 inhibited ROS production mimicking 15dPGJ$_2$ effects at all doses tested. In addition, treatment of AGs with PPARγ agonists including, troglitazone and ciglitazone also decreased ROS production, except for rosiglitazone agonist which did not show effect. Furthermore, the effect of 15dPGJ$_2$ on ROS production by AGs was reversed by PPARγ antagonist T007097 but not by GW9662. On the other hand, a transactivation assay was carried out in order to determine the signaling mechanism of 15dPGJ$_2$. We have demonstrated that both 15dPGJ$_2$ and its biotinylated analog activated the transcription of PPRE in presence of PPARα and PPARβ but not in the PPARγ. These results were confirmed by the study of the possible intectome of biotin-15dPGJ$_2$ by HPLC/MS analysis. Our data suggest that 15dPGJ$_2$ acts through both PPAR activation and covalent binding to proteins in gilthead seabream and identify for the first time a role for PPARα and PPARβ in the mechanism of action of 15dPGJ$_2$ in the resolution of the inflammation.

1. Introduction

Prostaglandins are small lipophilic molecules, with autocrine or paracrine activities, derived from cell membrane phospholipids through the action of phospholipase A_2 (PLA_2). Prostaglandin endoperoxide G/H synthase or homodimeric enzyme cyclooxygenase (COX) catalyzes the synthesis of PGH_2 from AA. Two different COX isoforms exist, the constitutive (COX-1) and the inducible (COX-2) isoforms. PGH_2 is converted into PGD_2 through a cell specific PGD_2 synthase (PGDS). The dehydration of PGD_2 within the cyclopentane ring yield PGJ_2, which can be transformed into Δ^{12}-PGJ_2 through an albumin-dependent manner, or into 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$) through a non-enzymatically dehydration (Hirata and Narumiya 2011a).

$15dPGJ_2$ is a cyclopentenone PG (cyPG) of the J series produced, in mammals, by mast cells or T cells, among others, and involved in different processes, including resolution of inflammation and control of cell proliferation (Harris et al. 2002b; Garzon et al. 2011). Although cyPG synthesis has been correlated with an increase of ROS production and with an up-regulated COX-2 mRNA levels (Garzon et al. 2011), it has been also reported that $15dPGJ_2$ plays an anti-proliferative and anti-inflammatory role at micromolar concentrations (Rajakariar et al. 2007a). Besides, it has been shown that this PG is involved in the switch from pro-inflammatory to anti-inflammatory activities (Martinez et al. 2012). Under inflammation conditions, it has been described an increase in $15dPGJ_2$ concentration, reaching from picomolar to nanomolar concentrations, although it is believed this concentration is underestimated due to its high biochemical instability (Garzon et al. 2011; Martinez et al. 2012) and to an active transport system used by this molecule to enter into cells (Narumiya and Fukushima 1986).

The cyPG $15dPGJ_2$ can signal through DP2 receptor, which belongs to the chemoattractant receptor family. Although DP2 binds selectively to PGD_2, $15dPGJ_2$ is also a selective and potent agonist of this receptor. DP2 activation is coupled to G_i protein leading to intracellular Ca^{2+} mobilization and inhibition of cAMP production (Hirata and Narumiya 2011a). Furthermore,$15dPGJ_2$ exerts its effects mainly through interaction with intracellular targets, including nuclear receptors as well as non-receptor proteins whose structure and function are modified by covalent binding to nucleophiles, such as, thiols groups of cysteine, lysine or histidine residues of proteins thiols (Perez-Sala 2011).

Best studied among the intranuclear targets of 15d-PGJ_2 are the peroxisome proliferator-activated receptors (PPARs) and the best studied effects of 15d-PGJ_2 in inflammation and immunity relate to activation of PPARγ. This receptor belongs to the nuclear hormone receptor (NHR) superfamily that act as ligand-activated transcription factors. Fatty acids and eicosanoids are natural analogs of PPARs. Once PPARs are activated form a heterodimer with RXR, a co-activator. This heterodimer binds to peroxisome proliferator response elements (PPRE) in the promoter regions of targets genes and elicits its expression. Three different PPAR isotypes have been characterized, α, γ and β/δ in mammals, birds

and amphibians (Willson et al. 2000; Leaver et al. 2005). Each isotype present different ligand specificity, biological activities and tissue expression (Zoete et al. 2007). Ligand binding domain consists in 34 residues highly conserve in human PPARs, but presents some differences that can be linked to the ligand affinity (Zoete et al. 2007). Unlikely, concerning to PPARs from fish species, much is still unknown, although some piscine PPARs have been described, including, three PPAR isotype, α, β and γ, from gilthead seabream (*Sparus aurata* L.) and plaice (*Pleuronectes platessa*) (Leaver et al. 2005). In silico analysis have shown the presence of several duplications for any PPAR genes in the several fish species like pufferfish (*Fugu rubripes*) and seabream which contains two homologs of the human PPARα (Maglich et al. 2003; Leaver et al. 2005), and zebrafish (*Danio rerio*) which genome has duplicate PPARα and PPARβ genes and a single gene for PPARγ (Bertrand et al. 2007; McPartland et al. 2007; Den Broeder et al. 2015). Different substances have been shown to be agonist of PPARγ such as, $15dPGJ_2$, the thiazolidinediones (TZD), including rosiglitazone, troglitazone and ciglitazone, specific PPARγ analogs. However, there are evidences that some PPARγ agonist exerts its effects through a PPARγ independent pathway (Ferreira-Silva et al. 2008; Schaaf 2017).

Nowadays, there are several studies reporting that $15dPGJ_2$ effects rely on PPARγ-independent mechanisms, however, almost nothing is known about the role of this CyPG in fish immunity and nothing about the signaling pathways involved in its activity. In a previous study we demonstrated that $15dPGJ_2$ decreases the respiratory burst of gilthead seabream acidophilic granulocytes (AGs) (see Chapter I), the main phagocytic cell type of this specie which is considered to be equivalent to mammalian neutrophils. Thus, the aim of this study is to go in depth into the role of $15dPGJ_2$ in gilthead seabream AGs functions as well as the signaling mechanisms through which exerts its actions.

2. Materials and Methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinoperygii, Sparidae) were bred and kept at the Oceanographic Centre of Murcia (Spain) in a 14 m^3 running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). Fish were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Isolation of phagocytes

AGs were obtained by MACS as described earlier (Roca et al. 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7)

(Sepulcre et al. 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100-200 µl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al. 2006).

2.3. Cell culture and treatments

Isolated AGs were stimulated for 16 hours at 23°C with 50µg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (*Va*DNA) (Pelegrin et al. 2004) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin (Biochrom). These PAMP concentration has been found to be optimal for in vitro activation of seabream AGs (Sepulcre et al. 2007a). Stimulated AGs were cultured with or without 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2, 1µM); biotinylated-5-deoxy-$\Delta^{12,14}$ PGJ_2 (Biotin-15dPGJ_2,1-10µM); the analog CAY10410 (CAY, 1-10µM); PPARγ agonists rosiglitazone (10 µM), troglitazone (5 and 10 µM) (Cayman Chemical) and ciglitazone (0,1 and 1 µM) (Santa Cruz Biotechnology); and PPARγ antagonists T0070907 (35 and 70 µM), GW9662 (35 and 70 µM) (Cayman Chemical) and Bisphenol A Diglycidyl Ether (BADGE) (2,5-25 µM) (Santa Cruz Biotechnology). In some experiments, PPARγ antagonists were added 30 minutes before add 15deoxyJ_2 or the PPARγ agonists. The mechanism of action of this PPARγ ligands is shown in Figure 2.

2.4. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, Amplification grade (1 unit/µg RNA, Life technologies). The SuperScrip III RNase H^- ReverseTranscripase (Life technologies) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1 µg of total RNA at 50 ºC for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the ribosomal protein S18 (*rps18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ for the constitutive expression analysis). The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice.

Gene	Accesion number	Name	Primer sequence (5'-3')
Il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
ptgs2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
Il10	FG261948	F	TGGAGGGCTTTCCTGTCAGA
		R	TGCTTCGTAGAAGTCTCGGATGT
PPARα	AY590299	F	TGCCTTCCTCTTGAACGCTT
		R	CAATGCTCTCCTGCATTCGC
PPARβ	AY590301	F	GCCATCATAGCCCCACTCTC
		R	ACGACTTGTCAACAGCCGAT
PPARγ	AY590304	F2	ATAGCGGTATGGACGCTGTG
		R2	TCCTGGATACTCTTGGCGA
rps18	AY587263	F1	AGGGTGTTGGCAGACGTTAC
		R1	CTTCTGCCTGTTGAGGAACC

Table 1. Gene accession numbers and primer sequences used for gene expression analysis.

2.5. Cell viability

Aliquots of cell suspensions were diluted in 200 µl PBS containing 40 µg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by flow cytometry.

2.6. Respiratory burst assays

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by AGs after stimulation for 16 hours (Mulero et al. 2001). This was done by adding 100 µM luminol and 1 µg/ml PMA (both from Sigma-Aldrich), while the chemiluminescence was recorded every 117 seconds for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of triplicate readings, expressed as the slope of the reaction curve from 117 to 1170 seconds, from which the apparatus background was subtracted.

2.7. Identification of biotin-15dPGJ2 interactome by mass spectrometry

Total leukocyte were obtained from gilthead seabream head kidney and stimulated for 16 hours at 23°C with 50µg/ml phenol-extracted genomic DNA from *V. anguillarum* ATCC19264 cells (*Va*DNA) (Pelegrin et al. 2004) in sRPMI as previously was described. Stimulated leukocytes were cultured with biotin-15deoxy-$\Delta^{12,14}$-PGJ_2 10µM for 2 hours in free FBS media. Cells were lysate with lysis buffer (50mM Tris-HCL [pH 7.5], 150mM NaCl, 1% Nonidet P40 and 1:20 protease inhibitor) and protein concentrations were estimated with the bicinchoninic acid protein assay reagent (Pierce) using BSA as a standard.

Lysates from bacterial DNA-stimulated leukocytes treated with or without biotinylated-15-deoxy-$\Delta^{12,14}$-PGJ_2 were incubated with 25 µl of Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen) at 4ºC for 2 h on a rolling shaker. The precipitates were washed three times with 1 ml of lysis buffer and once with PBS. The beads were resuspended in 30 µl of LDS sample buffer (Invitrogen) containing 10mM dithiothreitol and boiled at 100ºC for 5 minutes. Samples were then filtered with a 0.44-µm Spin-X filter (Corning) and alkylated with 50 mM iodoacetamide in 0.1 M NH4HCO3 at room temperature for 30 minutes in the dark and subjected to electrophoresis on a NuPAGE Bis-Tris 4–12% (w/v) polyacrylamide gel. Colloidal Coomassie stained gel was divided into pieces, and processed by CAID using as a protease trypsin. The resultant peptides were submitted to HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA). Data files were searched against the SwissProt Sparidae database.

2.8. Transactivation assay

All sea bream PPAR cDNAs were generously donate by Dr. M. Leaver (Leaver et al. 2005) and were cloned into pcDNA3 and verified by DNA sequencing. PPRE X3-TK-Luc plasmid was obtained from addgene (plasmid#1015). Human epithelial kidney 293T (HEK293T) cells were maintained in phenol-red Dulbecco's modified Eagle's medium (DMEM) high glucose (Biowest) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and L-glutamine (Biochrom). Forty-eight hours before transfection, HEK293T cells were harvested and seeded into 12-well plates at density of $0.65X10^5$ cells/well. For transfection, each well of adherent cells were co-transfected with 0.5µg of PPAR plasmids, 0.5µg of PPRE X3-TK-Luc (as a reported plasmid) and 1 µl of Lipofectamine (InvivoGen). Different DNA concentrations were equalized through the addition of empty vector DNA. Twenty-four hours after co-transfection, cells were cultured with different potential ligands of PPARs, perfluorooctanoic acid (PFOA, 100µM, Sigma), and palmitoleic acid (POA, 100µM, Cayman Chemical), 15deoxy-PGJ_2, (1µM, Cayman Chemical) and Biotin-15deoxy-PGJ_2 (10µM, Cayman Chemical). Twenty-four hours after add potential ligands, cells were washed once with PBS, lysate and firefly luciferase activity was quantified by commercial dual-luciferase reporter assay system, according to the manufacturer's instructions (Promega) in an FLUOstar Omega luminometer (BGM Labtech Instruments, Inc.). The protein concentrations of cell lysates were estimated with the bicinchoninic acid protein assay reagent (Pierce) using BSA as a standard. The results were represented as fold increase of luciferase luminescence with respect to the ethanol negative control. To express the results as fold increase, mock-transfected background was subtracted and results were normalized to total protein.

2.9. Statistical analysis

Data were analyzed by ANOVA and a Tukey's multiple range tests to determine differences between groups.

3. Results

3.1. Expression of seabream PPARs genes in AGs and modulation by PAMPs and 15dPGJ2

In mammals it is well stablished that one mechanism of action of $15dPGJ_2$ is as a PPARγ ligand. To ascertain whether $15dPGJ_2$ acts through PPARs in gilthead seabream, we first analyzed by RT-qPCR the constitutive expression of PPARs genes in AGs as well as its modulation by PAMPs and $15dPGJ_2$. It was found the expression of the genes coding for PPARα, PPARβ and PPARγ in non-stimulated AGs (Figure 1A) showing the PPARγ the highest transcript levels, followed by PPARβ and PPARα.

Notably, stimulation of AGs with genomic bacterial DNA resulted in increased mRNA levels of the gene coding for PPARγ (Figure 1D). However, the mRNA levels of *ppara* (Figure 1B) and *pparb* (Figure 1C) did not significantly changed upon *VaDNA* stimulation. In contrast, $15dPGJ_2$ was able to downregulate the mRNA levels of *pparg* induced in response to VaDNA (Figure 1D), while this PG had no effect in *ppara* and *pparb* ones (Figure 1B and 1C). These results could suggest a role for PPAR receptors in the resolution of the inflammation by $15dPGJ_2$ in gilthead seabream almost through the modulation of the genes encoding for them in stimulated AGs as well as by direct interaction with the PG.

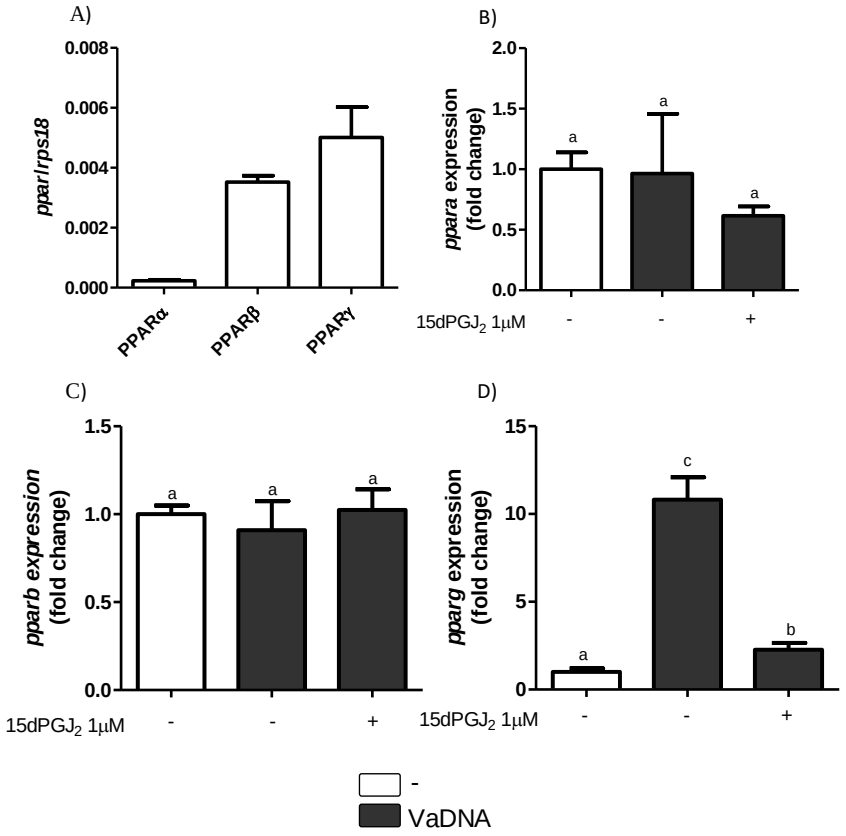

Figure 1. Constitutive expression of genes encoding for PPARs in AGs and modulation upon stimulation. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of $15dPGJ_2$ and with or without 50 mg/ml of VaDNA. The mRNA levels of the gene coding for PPARα (B), PPARβ (C) and PPARγ(D) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and expressed as the mean ± S.E. of the mRNA fold change in stimulated cells relative to non-stimulated cells. Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$).

3.2. 15dPGJ2 and biotinylated-5dPGJ2inhibit biological activity of AGs

Little is known to date concerning the role of cyPGs in fish immune responses. We have already reported an anti-inflammatory role for $15dPGJ_2$ by decreasing the expression of genes coding for cytokines and ROS production by AGs stimulated with VaDNA (see Chapter I). So far, nothing has been reported about the mechanism of action of cyPG in fish immunity. Another mechanism of action for cyPGs in mammals is by covalent protein modification through the formation of Michael adducts between cyPG and proteins (Figure 2B) (Stamatakis and Perez-Sala 2006). The biotinylated analog of $15dPGJ_2$ provides the possibility to explore the effects of this cyPG through protein modification but not by PPARγ interaction. The potent anti-inflammatory actions of cyPGs rely on the modification of key components of pro-inflammatory pathways (Diez-Dacal and Perez-Sala 2010). To get insight into the mechanism of action of cyPGs in seabream AGs, the effect of $15dPGJ_2$ and its biotinylated analog on ROS production and pro-inflammatory gene expression were analyzed. The results showed that, as

expected, treatment of stimulated AGs with physiological concentrations of 15dPGJ$_2$ resulted in decreased ROS production (Figure 3A), while cell viability was unaffected (data not shown). Additionally, 15dPGJ$_2$ dramatically downregulated the transcript levels of the genes coding for IL-1β and Ptgs2 (the limiting enzyme in PG production) in VaDNA-stimulated AGs (Figures 3B and 3C). Nevertheless, the biotinylated analog of 15dPGJ$_2$, at the same doses, neither affected ROS production nor mRNA levels of *ptgs2*, but slightly decreased de mRNA levels of *il1b* in AGs stimulated with VaDNA. However, treatment of VaDNA-stimulated GAs with higher doses of biotinylated-15dPGJ$_2$ resulted in decreased ROS production, in a dose dependent manner (Figure 3D), while cell viability was unaffected (data not shown). Together, these results indicate that 15dPGJ$_2$ has a potent anti-inflammatory effect which is mediated mainly through the binding to a specific receptor. Furthermore, covalent modification of proteins by 15dPGJ$_2$ has a weak contribution on its action almost at lower doses.

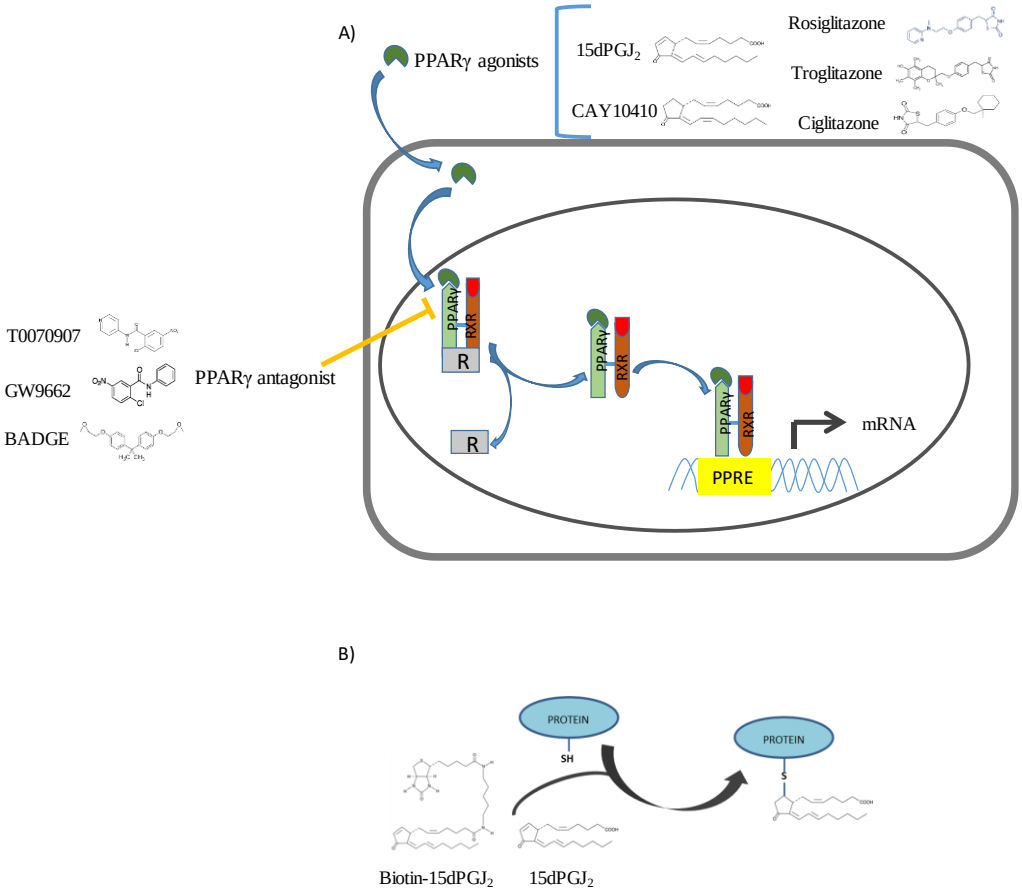

Figure 2. Diagram of 15dPGJ$_2$ signaling pathway and experimental approaches used in this study. 15dPGJ$_2$ can signal through PPARγ (A) or by covalent protein modification through the formation of Michael adducts between cyPG and proteins thiols groups of cysteine, lysine or histidine residues (B).

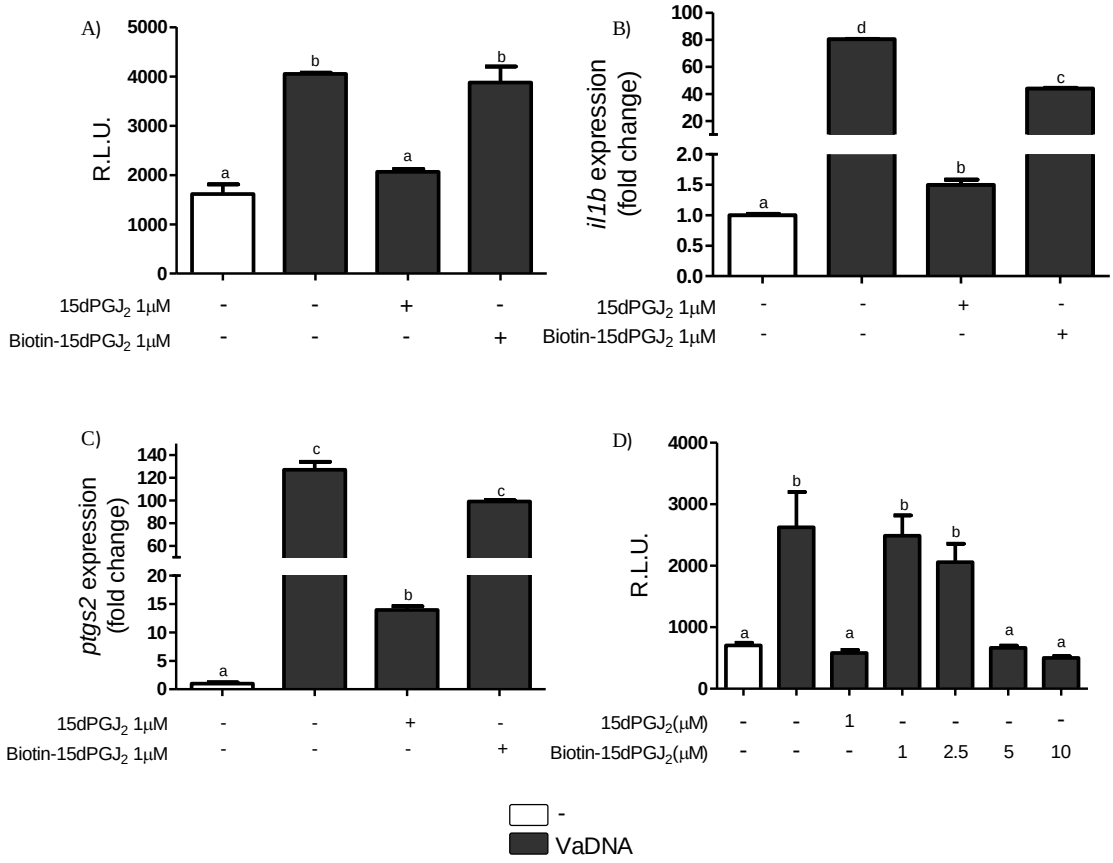

Figure 3. Anti-inflammatory effect of 15dPGJ$_2$ and biotinylated-15dPGJ$_2$ in ROS production by AGs upon stimulation. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of 15dPGJ2 or biotin-15dPGJ$_2$, with or without 50 mg/ml of VaDNA, and their respiratory burst activity was measured (A and D). The mRNA levels of the gene coding for IL-1β (B) and PTGS2 or COX2 (C) were determined by real-time RT-PCR. The gene expression is normalized against *rps18* and is shown as the mean ± S.E. of the mRNA fold change in stimulated cells relative to non-stimulated cells. Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$).

3.3. Agonists of PPARγ mimicked the effect of 15dGJ2 in seabream AGs

Experiments were conducted to address the potential PPARγ-dependent nature of the effect of 15dPGJ$_2$ in seabream AGs. In this set of experiments, the analog of 15dPGJ$_2$, 9,10-dihydro-15d-PGJ$_2$ (CAY10410), which could not undergo protein binding, but is a potent PPARγ agonist (Figure 2A), was used to compare its effect on the ROS production by stimulated AGs with the 15dPGJ$_2$ ones. The results showed that CAY10410 decreased ROS production mimicking the effect of 15dPGJ$_2$ at all doses tested (Figure 4A). These data are consistent with data obtained with the biotinylated analog of 15dPGJ$_2$ and support the idea that at lower doses this cyPG exerts its action through the binding to a specific receptor.

Another set of experiments was performed with different thiazolidinediones (TZD), such as, rosiglitazone, troglitazone and ciglitazone, which are selective PPARγ agonists (Figure 2A). It was found that troglitazone, but not rosiglitazone, decreased ROS production by VaDNA-primed AGs (Figure 4B) mimicking the effects of $15dPGJ_2$, while cell viability was unaffected (data not shown). Furthermore, the effect of troglitazone in the modulation of PPARγ expression was also analyzed. The results showed that treatment of stimulated AGs with troglitazone results in decreased mRNA levels of pparg (Figure 4D) in a similar way than $15dPGJ_2$ (Figure 1D). Furthermore, ciglitazone was also able to decrease ROS production by stimulated AGs, at all doses tested (Figure 4C). In the view of these results it is tempting to speculate that $15dPGJ_2$ could act through $PPAR_\gamma$ in seabream AGs, however it must be taken into consideration that not only cyPGs, but also other PPARγ agonists may display PPAR-independent effects.

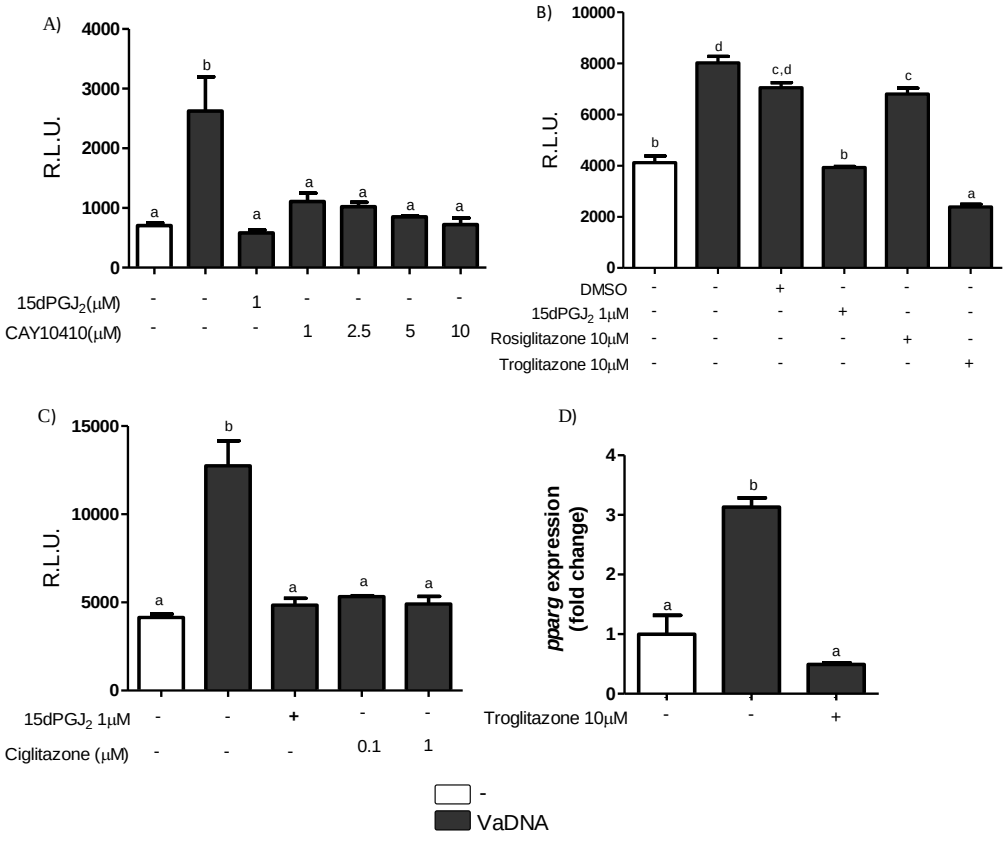

Figure 4. Effects of PPARγ agonist in AGs respitaroty burst upon stimulation. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of $15dPGJ_2$, CAY10410 (A), troglitazone, rosiglitazone (B) or ciglitazone (C) with or without 50 mg/ml of VaDNA, and their respiratory burst activity was measured. The mRNA levels of the gene coding for PPARγ (D) were determined by real-time RT-PCR in AGs treated with troglitazone. The gene expression is normalized against *rps18* and is shown as the mean ± S.E. of the mRNA fold change in stimulated cells relative to non-stimulated cells. Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$).

3.4. Effect of pharmacological inhibition of PPARγ in ROS production by 15dPGJ2 –treated AGs

The findings showed above prompted us to analyzed the ability of pharmacological inhibitors of PPARγ to block $15dPGJ_2$ effect in ROS production by stimulated AGs. The results showed that, PPARγ antagonist, T007097 reversed the effect of $15dPGJ_2$, but not troglitazone one, at all doses tested (Figure 5A). Strikingly, treatment of stimulated AGs with T007097 alone resulted in the upregulation of ROS production by these cells (Figure 5A). So the fact of blocking PPARγ ligands with the antagonist T007097 in stimulated conditions resulted in increased ROS production, highlighting the importance of this pathway in the regulation of the immune response in this context. However, due to the fact that T007097 was not able to block troglitazone effect it is tempting to speculate that the observed effect on $15dPGJ_2$ treatment, may be the result of the summative effect of both. Further experiments performed with the PPARγ antagonist, GW9662 showed that this compound partially reversed the effect of the PPARγ agonist troglitazone, in ROS production by stimulated AGs, but not $15dPGJ_2$ one (Figure 5B). Interestingly, unlike T007097, treatment of stimulated AGs with GW966 by itself did not have any effect in ROS production by these cells (Figure 5B). By contrast, PPARγ antagonist BADGE dramatically inhibited ROS production by stimulated AGs, showing an agonist activity (Figure 5C). Taken all together, the results obtained point to PPARγ-independent effects of PPARγ antagonist and/or agonist used in this study which implies a difficulty in order to get any conclusion when interpreting the results of pharmacological inhibition. So additional experiments are needed to determine the involvement of PPARγ in $15dPGJ_2$ signaling in seabream AGs.

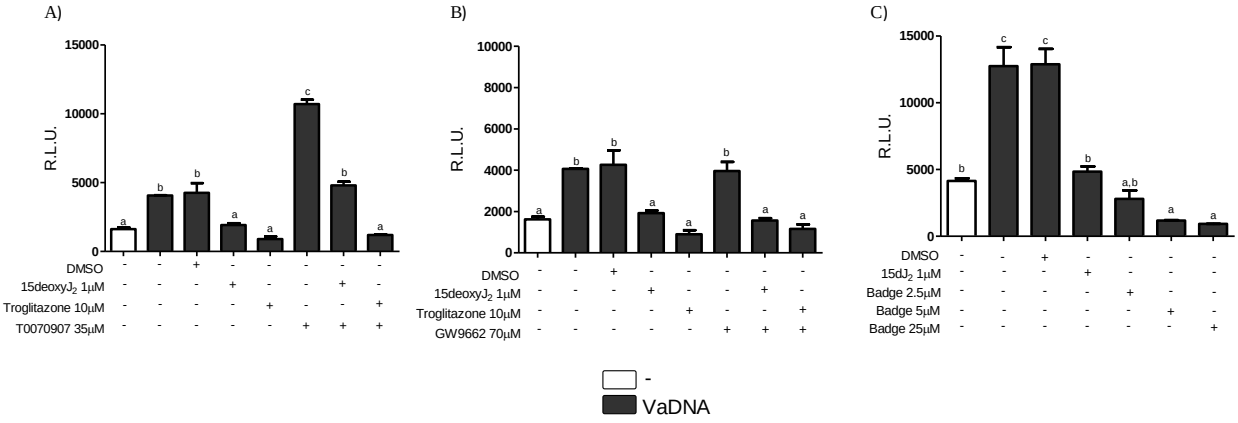

Figure 5. Effect of PPARγ antagonists in AGs activities upon stimulation. Purified AGs from head kidney were incubated for 16 h with the indicated concentrations of $15dPGJ_2$ or Troglitazone and with T0070907 (A), GW9662 (B) or BADGE (C) with or without 50 mg/ml of VaDNA. Antagonists were added 0,5 hours before add PPARγ agonists. The respiratory burst activity was measured. The results are represented as the mean ± S.E. Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$).

3.5. 15dPGJ2 signals through PPARα and PPARβ but not PPARγ interactions

In the view of the results exposed above, transactivation assays were performed to determine the ability of $15dPGJ_2$ to activate transcription through seabream PPARs in transient expression assays. A previous study has shown the capabilities of specific fatty acids of activating the transcription from a reporter construct with seabream PPARs (Leaver et al. 2005). Leaver and coworkers showed that palmitoleic acid (POA) exerts the largest effect in seabream PPARα and PPARβ and perfluoroctanoic acid (PFOA) elicits the highest response from PPARγ, but also from PPARα, albeit at lesser extent. Our results showed that $15dPGJ_2$ activated the transcription of the reporter construct in the presence of PPARα (Figure 6A). Furthermore, $15dPGJ_2$ activated the transcription with PPARβ although at lesser extent than POA (Figure 6B). Interestingly the biotinylated analog of $15dPGJ_2$ also activated the transcription with PPARα and PPARβ albeit at lower level (Figures 6A and 6B). Strikingly, in the presence of PPARγ, neither, $15dPGJ_2$ or its biotinyladed analog were able to activate transcription from the reporter construct used in this assay, while the positive control PFOA did so (Figure 6C). On the view of these results we concluded that $15dPGJ_2$ and biotin-$15dPGJ_2$ (at lesser extent) act through PPARα and PPARβ but not through PPARγ in gilthead seabream.

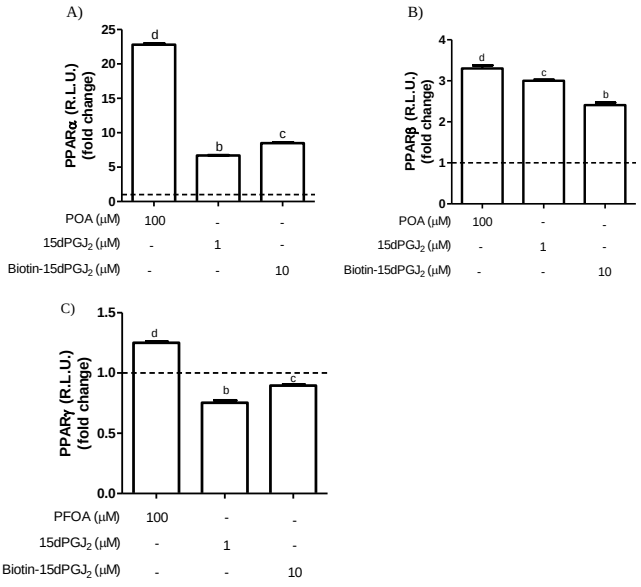

Figure 6. Transactivation of gilthead seabream PPARs. HEK cells were co-transfected with plasmids containing the seabream PPARα (A), PPARβ (B) and PPARγ (C) plasmids and with the reported plasmid. 24 hours post-transfection, cells were treated with palmitoleic acid (POA), perfluorooctanoic acid (PFOA) and $15dPGJ_2$ at the indicated concentrations. The results are shown as the mean ± S.E of fold increase in luciferase luminiscence respect to the ethanol negative control after mock transfected background subtraction and normalization to total protein. The results are representative of at least two independent experiments. Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$). The groups marked with "a" did not show statistically significant differences from control cells (indicated with horizontal dashed lines).

3.6. Biotin-15dPGJ2 covalent binds to PPARβ

The results exposed above prompted us to explore the existence of protein targets that could be selectively modified by $15dPGJ_2$ in seabream AGs by using the biotinylated analog. AGs stimulated or not were treated with or without biotin-$15dPGJ_2$. Streptavidin beads were used for precipitate proteins interacting with biotinylated $15dPGJ_2$ which were subjected to electrophoresis, Coomassie staining and HPLC/MS analysis. Interestingly, we noticed important quantitative and qualitative differences in $15dPGJ_2$-interactomes among VaDNA-stimulated and naive AGs (Figure 7). HPLC/MS revealed the interaction of the biotinylated $15dPGJ_2$ with PPARβ in protein extracts of VaDNA-stimulated AGs. These results indicate that covalent modification of PPARβ plays a key role in the mechanism of action in the anti-inflammatory effect exerted by $15dPGJ_2$ in seabream VaDNA-stimulated AGs, thus confirming data obtained by transactivation assays.

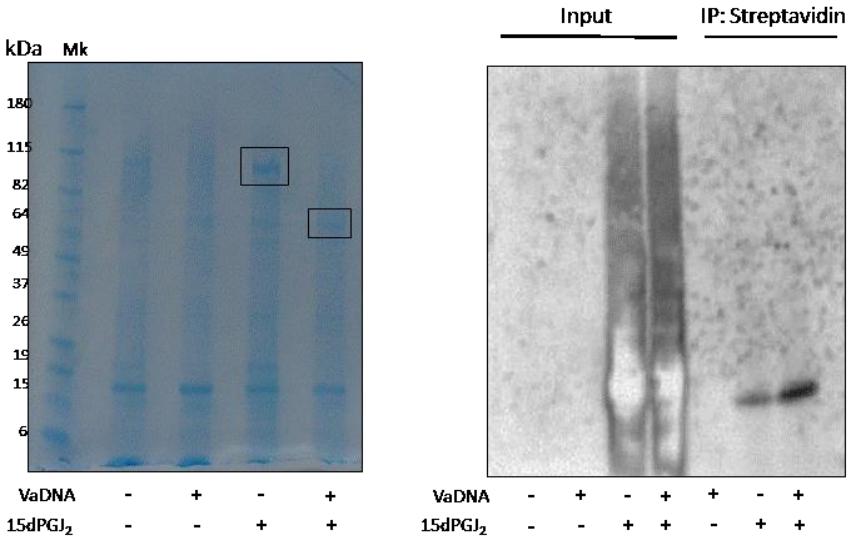

Figure 7. Interactions of biotin-$15dPGJ_2$ in challenged AGs. Total leukocyte were stimulated for 16 hours with VaDNA and cultured with biotin-$15dPGJ_2$. Inmnoprecipitation and Coomassie stained polyacrilamide gel were carried out. Gel was divided into pieces that were submitted to HPLC/MS to identify possible protein interactions with biotin-$15dPGJ_2$. Closed squares represents the stained gel region were appeared PPARβ peptides (A). The immunoprecipitates also were subjected to immunoblotting with the antibody Streptavidine-HRP (B).

4. Discussion

In mammals, it has been reported that cyPG are generated in increased amounts at the late stages of inflammation, playing, for instance, an anti-inflammatory role in the resolution of the inflammation (Gilroy et al. 2004). So far, little is known concerning the role of this prostaglandin in fish immune system. In a previous study we reported that $15dPGJ_2$ exerts an anti-inflammatory role by decreasing ROS production and the mRNA levels of the genes coding for IL-1β in seabream stimulated AGs.

Synthesis of 15dPGJ$_2$ has been related with the induction of *ptgs2* mRNA levels during the inflammation process (Oliva et al. 2003). By contrast, in this study we show that 15dPGJ$_2$ decreases the transcript levels of *ptgs2*, the main limiting enzyme of PG production, in seabream AGs treated with VaDNA.

Biological actions of cyPGs are partially due to their ability to covalently bind to cellular proteins. 15dPGJ$_2$ possesses electrophilic activity due to the presence of an α,β-unsaturated carbons within its cyclopentenone ring. These carbons are involved in the formation of Michael adducts with nucleophilic residues of proteins such as, cysteine, lysine or histidine amino acids (Domingues et al. 2013) or with cellular nucleophiles, such as, glutathione (GSH) (Garzon et al. 2011). This binding alters both, structure and function of proteins (Perez-Sala 2011). In particular, is the case of 15dPGJ$_2$ which covalently binds to a cysteine residue in the PPARγ ligand binding pocket through a Michael addition reaction, and this interaction is important to elicit the active conformation of the receptor (Shiraki et al. 2005; Waku et al. 2009). Structural requirements for covalent binding of 15dPGJ$_2$ to the PPARγ ligand domain are different from those involved in PPARγ-independent actions (Stamatakis and Perez-Sala 2006). 15dPGJ$_2$ occupy the ligand binding site in such a way that the carboxyl group the cyPG establishes interactions with the PPARγ backbone that are important for binding (Shiraki et al. 2005). CyPGs also may act through the interaction with the membrane receptor DP2. There is no evidence for covalent binding of cyPG to DP2. Currently, any orthologue of the mammalian DP2 receptor has not been annotated for any fish species which genome has been completely sequenced. So this study addressed the potential PPAR-dependent or –independent nature of the mechanism of action of 15dPGJ$_2$ in gilthead seabream immune response.

Three PPAR isotypes, α, β/δ and γ, has been characterized in mammals, with specific tissue expression, different ligand specificity and hence with different biological activities (He et al. 2012). Orthologues of these receptors have been identified in several fish species (Bertrand et al. 2007; McPartland et al. 2007; Den Broeder et al. 2015). Those piscine PPARs show different ligand binding properties and tissue distribution from their mammalian counterparts (Leaver et al. 2005). By contrast PPAR-dependent transcriptional activation in fish involves very similar promoter structural requirements as in mammals (Leaver et al. 2005). Nowadays, little it is known about the role of PPARs in the regulation of fish immunity. It has been demonstrated faint products and no significant increase in PPARγ expression in seabream head kidney upon intraperitoneal injection of LPS stimulation (Acerete et al. 2007). Moreover, 15dPGJ$_2$ is able to alter the expression of both PPARα and PPARγ in zebrafish (Ibabe et al. 2005), to decrease *pparb* and *pparg* levels in differentiated adipocytes of red sea bream (Oku and Umino 2008) and to induce *pparb* expression in grass carp (*Ctenopharyngodon idella*) (He et al. 2012). In this study we have reported the expression of *ppara,pparb* and *pparg* in seabream AGs, the main phagocytic cell type of this specie and the up-regulation of *pparg* levels upon VaDNA-stimulation, effect that was reversed by 15dPGJ$_2$ treatment. These data constituted the basis that allowed

us to go further the mechanism of action of the cyPG in seabream AGs. In this study, the potential PPAR-dependence of the effects of $15dPGJ_2$ on those cells has been addressed by using several approaches: i) comparison of the effects of $15dPGJ_2$ with those of two $15dPGJ_2$ analogs (biotinylated $15dPGJ_2$ that has not the ability to activate mammalian PPARγ, and CAY 10410 which lacks a double bound within the cyclopentenone ring and hence, lacks the ability to covalent bind to proteins, but retains the ability to bind to PPARγ; ii) comparison of the effects of $15dPGJ_2$ with those of other PPARγ agonists, such as rosiglitazone, troglitazone and ciglitazone; iii) assessment of the ability of PPARγ antagonists, such as T00709, GW9662 and BADGE, to block $15dPGJ_2$ effects. A schematic picture of those approaches is showing in Figure 2.

Several reports outline those approaches. It has been reported that CAY10410 did not mimic the effect of $15dPGJ_2$, which increase neuronal cell death in rat (Liu et al. 2013), suggesting the toxicity of $15dPGJ_2$ is mediated through covalent binding to proteins in this context. Similarly, $15dPGJ_2$ inhibits transforming growth factor beta (TGFβ) signaling in human pulmonary fibrosis, while CAY10410 did not, pointing out that the role of $15dPGJ_2$ is mediated through covalent binding to proteins (Kulkarni et al. 2011). However, in human colorectal carcinoma cells, both $15dPGJ_2$ and CAY10410 reduce CXCR4 expression, although $15dPGJ_2$ has a more potent effect than CAY10410 (Richard et al. 2007), suggesting that this cyPG signals both through PPARγ activation and covalent binding to proteins. On the other hand, the biotinylated analog of $15dPGJ_2$, is able to form adducts with proteins, although, due to the volume of the biotin group added to the C terminal do not activate PPARγ (Stamatakis and Perez-Sala 2006), but binds selectively to proteins such as, NF-kappaB (Perez-Sala et al. 2002; Gayarre et al. 2007), which is inhibited or H-RAS, iNOS, and COX2, which are activate by biotin-$15dPGJ_2$ (Oliva et al. 2003; Stamatakis and Perez-Sala 2006). In this study, we have found that both structural analogs of $15dPGJ_2$, the biotinylated one and CAY10410 mimicked the effect of $15dPGJ_2$ on ROS production by seabream stimulated AGs. However, doses of biotin-$15dPGJ_2$ needed to reach the same effect than$15dPGJ_2$ was 10 times higher than $15dPGJ_2$, or CAY10410. Those results suggest a prevalence of the PPAR-dependent nature of the effects of $15dPGJ_2$ on seabream AGs on the –independents ones. Consistent with our data,$15dPGJ_2$ activates PPARs at lower doses and its toxic effect at higher doses is eliminated by activating the detoxification mechanisms into the cells by covalent protein modification (Gayarre et al. 2007).

Although TZD such as, rosiglitazone, troglitazone and ciglitazone, has been described as mammalian and fish specific PPARγ agonists (Guan and Breyer 2001; Balakumar et al. 2007; Richard et al. 2007; Niemoeller et al. 2008; Tingaud-Sequeira et al. 2011; Gupta et al. 2015; Ouadah-Boussouf and Babin 2016; Nazim et al. 2017), our results have shown that troglitazone and ciglitazone but not rosiglitazone mimics the effect of $15dPGJ_2$ downregulating ROS production by VaDNA AGs. These results are consistent with data obtained by M. Leaver and coworkers, showing a weak PPARγ, and

much lesser PPARα activation by rosiglitazone which could be due to the structural differences between human and seabream PPARγ, specifically due to differences in the ligand binding domain (Leaver et al. 2005; Schaaf 2017). Surprisingly, although BADGE has been broadly defined as PPARγ antagonist (Wright et al. 2000; Song et al. 2009), we have found that in our context, this compound acts as a PPARγ agonists at all doses tested. This data are consistent with a previous study where BADGE showed a PPARγ agonist effect (Bishop-Bailey et al. 2000). On the other hand, T007097 and GW9662 have been described as PPARγ antagonists being able to block $15dPGJ_2$ effects (Richard et al. 2007) or PPARγ agonists ones (Song et al. 2009; Shafizadeh et al. 2014; Nazim et al. 2017). Our data showed that T007097 increased ROS production by VaDNA stimulated AGs and abolishes the effect of $15dPGJ_2$ in this context, however did not exerted any effect in the activity of the PPARγ agonist troglitazone. So it is tempting to speculate that neutralization of the effect of $15dPGJ_2$ by T007097 may be the result of two independent events. Furthermore, the PPARγ antagonist GW9962 blocked partially the effect of troglitazone in the inhibition of ROS production by AGs but in keeping with previous studies (Katura et al. 2010), failed to do so those $15dPGJ_2$. Nevertheless, in the view of the results showing PPARα and PPARβ, but not PPARγ activation by $15dPGJ_2$ and its biotinylated analog, we demonstrate here in that the PPARγ similar effects showed in this study are due to PPARγ independent pathways, as previously described V. Ferreira-Silva (Ferreira-Silva et al. 2008) and explain the fact that, the PPARγ antagonist, GW9962, failed to block $15dPGJ_2$ effects.

It has been reported that PPARs possess ligand specificity. PPARα is activated by fibrates and polyunsaturated fatty acid such as, eicosanoids (Guan and Breyer 2001), PPARβ is activated by linoleic acid, arachidonic acid, eicosapentenoic acid (EPA) and PGA_1 (Guan and Breyer 2001) and PPARγ is activated by PGA_2, PGD_2, PGJ_2 and $15dPGJ_2$ (Yu et al. 1995; Bishop-Bailey et al. 2000; Guan and Breyer 2001; Park and Christman 2006). However, the major finding in our study is that $15dPGJ_2$ is able to activate seabream PPARα and β, but not PPARγ. Moreover, using mass spectrometry approach we have found that this cyPG interact with seabream PPARβ by covalent binding in stimulated AGs. Taken together, all our data point out that, like in mammals, $15dPGJ_2$ acts through both PPAR activation and covalent binding in the gilthead seabream. By contrast, although this cyPG has been classically described as a specific ligand of PPARγ in mammals (Corton et al. 2000), the PPARs involved in the anti-inflammatory role of $15dPGJ_2$ in this fish species are PPARα and β. The results obtained in this study place the activation of PPARγ by $15dPGJ_2$ in a most evolutionary advanced mechanism of action of cyPGs.

CONCLUSIONS

1. Two orthologues of the mammalian L-PGDS has been described in gilthead seabream (PGDS1 and PGDS2). The mRNA levels of *pgds1* are increased in acidophilic granulocytes upon stimulation with different PAMPs and its regulation correlates with PGD_2 synthesis. Whereas *pgds2* is not expressed in those cells.
2. PGD_2 (16,16-dimethyl PGD_2 and 11-deoxy-11-metilen-PGD_2) and the series J_2 of prostaglandins (Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2) exert an anti-inflammatory effect, by decreasing ROS production and mRNA levels of *il1b* and *il10*, in stimulated acidophilic granulocytes.
3. The amounts of PGE_2 in gilthead seabream are mainly regulated by PGE_2 synthesis rather than by its catabolism, both in vivo and in vitro.
4. PGE_2 promotes the M2 polarization of gilthead seabream macrophages increasing the mRNA levels of *il10*, *mrc1* and *arg2*.
5. PGE_2 signals through a cAMP/PKA/CREB pathway in macrophages.
6. PGE_2 and its stable analog, 16,16-dimethyl-PGE_2, inhibit the biological function of acidophilic granulocytes upon stimulation with VaDNA, through the inhibition of ROS production and the expression of *il1b*, *il10* and *il6*.
7. 15-deoxy-$\Delta^{12,14}$-PGJ_2 signals through the interaction with PPARs (PPARα and PPARβ) and by covalent binding to proteins in gilthead seabream immune response.
8. Seabream PPARβ is a target of biotinylated-15-deoxy-$\Delta^{12,14}$-PGJ_2 in stimulated acidophilic granulocytes.

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RESUMEN EN CASTELLANO

En un mundo poblado por más de 7 millones de personas y que según la FAO (2018) en 2050 estará habitado por más de 9 millones de personas, uno de los grandes problemas a resolver es la búsqueda de alimentos. En 2015, el 17% de la ingesta mundial de proteína era de origen marino y en 2016, el 47% de la producción pesquera procedía de la acuicultura. En los últimos años, la acuicultura ha experimentado un marcado incremento sobre la pesca extractiva. Esto supone, que según la FAO en el año 2030 más del 65% de la producción pesquera procederá de la acuicultura. La acuicultura consiste en la producción tanto de animales como de plantas acuáticas usando diferentes métodos y técnicas para mejorar su eficiencia. Esto supone que los animales estén sometidos a manipulación, confinados en un espacio reducido, lo cual genera estrés en el animal. Bajo estas condiciones se favorece el desarrollo de enfermedades infecciosas que son causa de grandes pérdidas económicas. Por ello, el conocimiento del sistema inmunitario de peces, y en particular, de las especies de importancia económica para la acuicultura en España se ha convertido en uno de los objetivos principales de investigación acuícola. Además, el estudio del sistema inmunitario de peces es interesante desde el punto de vista científico básico, dado que los peces teleósteos ocupan una posición clave desde un punto de vista evolutivo ya que representan el primer grupo de vertebrados que presentan sistema inmunitario innato y sistema inmunitario adaptativo (basado en rag). El sistema inmunitario innato está constituido por barreras físicas y químicas, efectores celulares (leucocitos) y componentes humorales (lisozima, lectinas, inhibidores de crecimiento, etc.). El sistema inmunitario adaptativo está compuesto por componentes celulares (linfocitos T y B) y componentes humorales (anticuerpos, Ac). A pesar de que el conocimiento del sistema inmunitario de peces presenta cierta homología con el sistema inmunitario de vertebrados superiores, existen muchas diferencias entre ambos. Una de las diferencias más destacadas es la ausencia de médula ósea en peces, siendo el riñón cefálico el órgano hematopoyético por excelencia. Otra importante diferencia con los vertebrados superiores es que, en peces, el sistema inmunitario depende básicamente de los mecanismos de la defensa innata en contraposición con el gran desarrollo y potencia de la defensa adaptativa en vertebrados superiores. La dorada (*Sparus aurata* L.), es un pez teleósteo extensamente usado como modelo animal de investigación de la respuesta inmune de peces y es una especie de interés económico importante para la acuicultura mediterránea. En la dorada, el riñón cefálico está constituido por células precursoras y por tres poblaciones de leucocitos que son macrófagos, linfocitos y granulocitos acidófilos (GAs). Los GAs, junto con los macrófagos, son los principales efectores de la respuesta inmunitaria innata en la dorada. Además, estas células pueden producir especies reactivas del oxígeno (ROS) como mecanismos de defensa frente a patógenos. Diversos estudios realizados en dorada han descrito a los GAs como las células fagocíticas más activas y abundantes en esta especie y son considerados como las células equivalentes desde un punto de vista funcional a los neutrófilos de mamíferos debido a su potente actividad fagocítica y gran capacidad de producción de ROS y citoquinas, así como por la presencia de un amplio espectro de receptores tipo Toll (TLRs) (Sepulcre et al. 2002; Sepulcre et al. 2007). A pesar de que el sistema inmunitario de los peces teleósteos ha sido ampliamente estudiado durante décadas, aún hay mucho por investigar, especialmente aquello

que concierne al papel de las prostaglandinas (PGs) y sus rutas de señalización. Sin embargo, existen pocos estudios referentes al papel de las PGs en los fagocitos de peces. Así, el objetivo de la presente tesis es dilucidar el papel de las PGs en la respuesta inmunitaria de la dorada.

Los eicosanoides (Prostaglandinas (PGs), Tromboxanos (TXs), Prostaciclinas (PCs), Leucotrienos (LTs), Lipoxinas (LPs) y endocanabinoides) son mediadores lipídicos que poseen un papel importante en la respuesta inmunitaria. A su vez, PGs, LTs y PCs son conocidos como prostanoides. Los prostanoides son mediadores lipídicos derivados del AA que es liberado de la membrana celular por la acción de la enzima fosfolipasa A_2 (PLA_2) en respuesta a diferentes estímulos, por ejemplo, citoquinas. A continuación, la enzima ciclooxigenasa (COX, también conocida como prostaglandina G/H sintasa o prostaglandin-endoperóxido sintasa 2) convierte el AA en PGH_2. Esta PG es convertida en los distintos postanoides mediante reacciones espontáneas o reacciones enzimáticas (Introducción, Figura 2).

Las PGs son moléculas de pequeño tamaño derivadas de ácidos grasos poliinsaturados procedentes de los fosfolípidos de membrana celular. Existen dos series de PGs, las PGs de la serie 2, cuyo precursor es el ácido araquidónico (AA) y las PGs de la serie 3 cuyo precursor es el ácido eicosapentanoico (EPA). En la ruta de biosíntesis de las PGs existen dos pasos limitantes, el primero es la disponibilidad de los ácidos grasos precursores de PGs (AA y/o EPA) y el segundo es la conversión del AA mediada por la enzima COX. En mamíferos existen, dos isoformas de la enzima COX, la isoforma constitutiva (COX-1) que se expresa en diferentes tipos celulares; y la isoforma inducible (COX-2) cuya expresión aumenta durante la inflamación (Simmons et al. 2004). Esta enzima convierte el AA o EPA en PGH_2 o PGH_3 respectivamente. Esta conversión se trata de una reacción en dos etapas. La PGH_2 se convierte en PGE_2 por la acción de la enzima PGE sintasa (PGES) (Joo and Sadikot 2012). En mamíferos existen distintas isoformas de esta enzima, la isoforma citosólica (cPGES) y la microsomal (mPGES) (Murakami et al. 2002). Por otro lado, la PGH_2 también puede ser convertida en PGD_2 por la acción de la PGD sintasa (PGDS). En mamíferos, se han descrito dos tipos de PGDS, la forma hematopoietica (H-PGDS) y la forma lipocalina (L-PGDS). Finalmente, la PGD_2 es convertida en derivados ciclopentenonas (CyPGs) tales como, 15-deoxi-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2) y $\Delta^{12}PGJ_2$, mediante reacciones independientes o dependientes de albúmina, respectivamente (Introducción, Figura 4) (Shibata et al. 2002). Los efectos de las PGs dependen de diversos factores tales como, el microambiente, el tipo celular, el perfil de expresión de los distintos receptores de PGs y de la afinidad por el ligando (Park and Christman 2006).

En mamíferos, se han descrito diversos efectos anti y pro-inflamatorios para la PGE_2 dependiendo del tipo de receptor al que se una y, por lo tanto, de la ruta de señalización activada (Harris et al. 2002; Harizi et al. 2003; Kabashima et al. 2003; Kim et al. 2010; Wright et al. 2010). Del mismo

modo, la PGD_2 posee un papel anti o pro-inflammatorio dependiendo del tipo de receptor activado (Park and Christman 2006; Hammad et al. 2007; Ricciotti and FitzGerald2011). Sin embargo, a las CyPGs se les ha atribuido un papel clásicamente anti-inflamatorio, pro-apoptótico y anti-proliferativo (Garzon et al. 2011).

En mamíferos, las PGs señalizan a través de receptores de la superficie celular que presentan en su estructura siete dominios transmembranales tipo rodopsina y están acoplados a proteínas G (GPRs). Estos receptores constituyen la subfamilia de receptores de prostanoides (Hata and Breyer 2004). Por el contrario, los derivados ciclopentenonas de las PGs (CyPGs) no poseen receptores de superficie celular (Negishi and Katoh 2002).

La subfamilia de receptores de prostanoides está constituída por nueve miembros, DP, CRTH2 o DP2, EP1-4, FP, IP y TP, a su vez divididos en 3 grupos o subfamilias de receptores en función de la similitud en su estructura y de la ruta de señalización que desencadenan (Introducción, Figuras 3 y 4). Estos receptores se denominan en función del prostanoide al que se unen con mayor afinidad: DP y DP2 son receptores de la PGD_2; EP1-4 son receptores de la PGE_2; FP es el receptor de $PGF_{2\alpha}$; IP es el receptor de la PGI_2 o PC; y, por último, TP es el receptor de TXA_2 (Toh et al. 1995; Breyer and Breyer 2001; Breyer et al. 2001; Hata and Breyer 2004; Jabbour and Sales 2004; Hirata and Narumiya 2011; Hirata and Narumiya 2012).

Los receptores IP, DP, EP2 y EP4 constituyen la subfamilia de receptores relajantes, denominados así porque producen la relajación del músculo liso. La activación de estos receptores produce la inducción de la enzima adenilil ciclasa (AC) dando lugar a la producción intracelular de adenosina monofosfato cíclico (cAMP). La subfamilia de receptores contráctiles está compuesta por los receptores TP, FP y EP1, denominados así porque producen la contracción del músculo liso. Estos receptores una vez activados producen la movilización de Ca^{2+} citosólico. El receptor EP3, también conocido como receptor inhibidor, dependiendo de la variante expresada y activada puede actuar como receptor relajante y como receptor contráctil. Por último, el receptor DP2 produce la inhibición de los niveles de cAMP y la movilización del Ca^{2+} (Introducción, Figuras 3 y 4) (Hata and Breyer 2004; Jabbour and Sales 2004; Hirata and Narumiya 2011; Hirata and Narumiya 2012).

La PGD_2 puede interaccionar con los receptores DP1 y DP2 con prácticamente la misma afinidad, produciendo distintos efectos dependiendo del receptor activado. Sin embargo, ambos receptores también presentan elevada afinidad por PGJ_2 y por $15dPGJ_2$, respectivamente (Hirai et al. 2001; Sawyer et al. 2002).

La PGE_2 se une de forma específica a los receptores EP, pero presenta distintos efectos biológicos dependiendo del receptor activado. Además, los distintos receptores presentan distintas

afinidades por la PGE_2, habiéndose observado que EP3 y EP4 poseen mayor afinidad por dicha PG que EP1 y EP2 (Abramovitz et al. 2000).

La $15dPGJ_2$ y la Δ^{12}-PGJ_2 son CyPGs que pueden atravesar la membrana celular mediante un sistema de transporte (Harris et al. 2002, Negishi and Katoh 2002). La $15dPGJ_2$ es capaz de ejercer sus efectos a través de la interacción con receptores (PPARγ o DP2) o mediante la interacción con grupos tiol de otras proteínas. PPARγ pertenece a la superfamilia de receptores nucleares y está asociado a procesos de inflamación y al metabolismo lipídico. Tras la activación de PPARγ, éste receptor se une al receptor X retinoide (RXR) para formar un heterodímero que será translocado al interior del núcleo celular donde se unirá a elementos de repuesta (PPREs). Estos PPREs están localizados en la región promotora de los genes diana (Kliewer and Willson 1998). DP2 activado produce la inhibición de los niveles de cAMP y la movilización de Ca^{2+}. Por otra parte, esta CyPG posee dos carbonos β en su estructura que le otorgan una elevada reactividad. Estos carbonos pueden ser atacados por grupos tiol de otras proteínas formando así aductos covalentes de Michael y modificando tanto la estructura como la función de la proteína.

Los principales componentes de la membrana celular en peces son AA, ácido eicosapentaenoico (EPA) y ácido docoxahexaenoico (DHA) (Rowley et al. 1995). Por otro lado, diversos estudios realizados en peces han evidenciado la presencia de homólogos de la enzima COX-2 de mamíferos, entre los que cabe destacar, los estudios realizados en dorada (*Sparus aurata*, L.) (Sepulcre et al. 2007; Mulero et al. 2008; Sepulcre et al. 2011; Lopez-Muñoz et al. 2012; Boltana et al. 2014). Así mismo, diversos estudios realizados en peces teleósteos han evidenciado la presencia de homólogos de mPGES-1 y c-PGES (Pini et al. 2005); homólogos de PGDS (Bayne et al. 2001; Boltaña et al. 2011). Sin embargo, muy pocos estudios están enfocados en la correlación de la presencia de estas enzimas implicadas en la ruta de biosíntesis de las PGs y la producción de PGs en peces.

Los objetivos de la presente tesis doctoral son:

1. Determinar la caracterización molecular de la prostaglandina D sintasa (PGDS) de la dorada (*Sparus aurata*, L.)
2. Estudiar el perfil de expresión de los diferentes genes implicados en el metabolismo de las prostaglandinas en dorada *in vitro* e *in vivo*.
3. Analizar el papel de las distintas prostaglandinas (PGE_2, PGD_2, $15dPGJ_2$ and Δ^{12}-PGJ_2) en las principales funciones biológicas de los fagocitos de dorada (macrófagos y granulocitos acidófilos).
4. Determinar la ruta de señalización implicada en la actividad de la PGE_2 y la $15dPGJ_2$ en macrófagos y granulocitos acidófilos de dorada respectivamente.

La investigación de la Tesis Doctoral se ha llevado a cabo secuencialmente en tres capítulos:

En el primer capítulo, anotamos y caracterizamos molecularmente dos genes de la L-PGDS de mamíferos, PGDS1 y PGDS2, en dorada. Asimismo, estudiamos los perfiles de expresión génica. PGDS1 presenta dos motivos ATTTA en el extremo 3', ausentes en PGDS2, y que parecen ser responsables de la inestabilidad de los mRNAs de citoquinas de mamíferos y peces. Ambos genes, presentan un único marco de lectura que da lugar a polipéptidos de 184 y 180 aminoácidos respectivamente y que muestran una elevada similitud con la enzima Prostaglandina D sintasa (PGDS) tipo lipocalina de mamíferos (42.8% para PGDS1 y 46.1% para PGDS2). A pesar de que ambas proteínas presentan un dominio lipocalina, carecen de la cisteína esencial para la actividad de síntesis de PGD_2 en la L-PGDS de mamíferos, al igual que ocurre con la L-PGDS de pez cebra y trucha arcoíris.

A continuación, se analizaron los niveles de mRNA de ambos genes mediante RT-qPCR en muestras de GAs estimulados *in vitro* con distintos PAMPs (DNA bacteriano procedente de *Vibrio anguillarum* (VaDNA) y flagelina). Los resultados mostraron que la estimulación de los GAs resultó en un incremento de los niveles de *pgds1* a lo largo del tiempo analizado. Del mismo modo, los niveles de *ptgs2* y de *il1b* también experimentaban un incremento, alcanzando su máximo a 1 día post-estimulación, el tiempo más corto analizado. Sorprendentemente, *pgds2* no se expresó en condiciones basales ni en GAs estimulados.

A la vista de estos resultados analizamos la producción de PGD_2 en GAs estimulados mediante ELISA. Los resultados mostraron que la estimulación de dichas células resulta en un incremento en la producción de PGD_2 con una cinética similar a la expresión de *pgds1*.

A continuación, se determinó el efecto de la PGD_2, en las principales funciones de los GAs, tales como, la producción de especies reactivas de oxígeno (ROS) y de citoquinas. Para ello se utilizaron dos análogos estables de la PGD_2 (16,16-dimetil-PGD_2 y 11-deoxi-11-metilen-15-keto-PGD_2) que señalizan a través de los receptores DP1 y DP2, respectivamente. Los resultados mostraron que ambos análogos de la PGD_2 reducían la producción de ROS, aunque únicamente la 16,16-dimetil-PGD_2 reducía los niveles de transcripción de *il1b* por los GAs. Del mismo modo se estudió el efecto de CyPGs, encontrándose que tanto la $15dPGJ_2$ como la Δ^{12}-PGJ_2 reducían la producción de ROS y de *il1b* en estas células.

Finalmente se analizaron los niveles de mRNA de los genes que codifican para PGDS1 y PGDS2, mediante RT-qPCR, en diferentes tejidos de dorada retadas o no con el patógeno *Vibrio anguillarum.* Los resultados mostraron que ambos genes se expresaban en todos los tejidos analizados y que los niveles de expresión de *ptgs2*, *pgds1* y *pgds2* se modulaban en los distintos tejidos inmunitarios tras el reto.

Conjuntamente estos resultados revelan la existencia de dos ortólogos de la enzima L-PGDS en dorada, PGDS1 y PGDS2, sin embargo, no se encontró ningún ortólogo de la enzima H-PGDS en peces teleósteos. Los genes que codifican para ambas enzimas se expresan de forma constitutiva en todos los tejidos analizados y su expresión se ve modulada tras el reto bacteriano, sin embargo, su distribución tisular nos sugiere que ambos ortólogos podrían tener diferentes funciones. Al mismo tiempo, los GAs estimulados con distintos PAMPs producen PGD_2, siendo la enzima PGDS1 la candidata implicada en su síntesis ya que no se detectó expresión *pgds2* en dichas células ni de forma constitutiva ni tras la estimulación con distintos PAMPs. Además, la PGD_2 y sus derivados CyPGs poseen un efecto anti-inflamatorio mediante la inhibición de la producción de ROS y citoquinas. Estos resultados sugieren un papel clave de dicha PG en la resolución de la inflamación en GAs de dorada.

En el segundo capítulo estudiamos el papel de la PGE_2 en la respuesta inmunitaria de dorada *in vivo* e *in vitro*. Los niveles de mRNA de los genes implicados en el metabolismo de la PGE_2 en distintos tejidos, así como su modulación tras el reto con *V.anguillarum* fueron analizados mediante RT-qPCR. Los resultados mostraron que *pges* (implicado en la síntesis de PGE_2) se expresaba en todos los tejidos analizados, observándose mayores niveles de este gen en los órganos inmunitarios. Además, se observó que tras el reto con *V. anguillarum*, los niveles de *pges* incrementaban principalmente en los órganos inmunitarios. Sin embargo, los niveles de *hpgd* (implicado en su degradación), fueron menores en los tejidos inmunitarios de doradas sometidas al reto. Además, se analizaron, mediante RT-qPCR, la expresión de los genes implicados en el metabolismo de la PGE_2 en células fagocíticas, así como su modulación tras la estimulación con distintos PAMPs. Los resultados mostraron que la estimulación de los macrófagos y de GAs resultó en un incremento de los niveles de mRNA de *ptgs2* y *pges* con la misma cinética, alcanzándose el máximo valor 3h post-estimulación. Sin embargo, los niveles de *hpgd* apenas estaban inducidos en fagocitos estimulados.

Posteriormente se estudió el efecto de la PGE_2 en macrófagos estimulados mediante un análisis de la expresión de algunos de los principales genes implicados en la respuesta inmunitaria. Los resultados obtenidos mostraron que el tratamiento con PGE_2 de macrófagos estimulados incrementaba la expresión de *il10*, pero no modulaba la expresión de *il1b*. Dado que la producción de IL-10 es un marcador del fenotipo M2 de macrófagos, se procedió al análisis de la expresión de otros marcadores de dicho fenotipo, tales como el receptor de manosa tipo c (MRC1) y arginasa 2 (ARG2). Los resultados mostraron que la PGE_2 incrementaba los niveles de *mrc1* y de *arg2*. También se analizó el efecto de la PGE_2, así como de un análogo estable (16,16-dimetil-PGE_2) en GAs estimulados con VaDNA, resultando en una inhibición de la producción de ROS y una reducción de los niveles de mRNA de *il1b*, *il10* e *il6* con ambos tratamientos. Finalmente se analizó la ruta de señalización de la PGE_2 en macrófagos de dorada. Para ello, se utilizó un análogo permeable de cAMP que es capaz de activar PKA (dbcAMP). Los resultados mostraron que dbcAMP imitaba los efectos de la PGE_2 en macrófagos

estimulados, pero no en GAs. Estos resultados sugieren que dicha PG señaliza a través de la ruta cAMP/PKA/CREB en macrófagos de dorada.

En conjunto, estos resultados nos indican que la PGE_2, cuya concentración podría estar controlada principalmente por la expresión de los genes que regulan su biosíntesis, podría desarrollar un papel importante en la regulación de la inflamación en dorada. Esta PG ejercería su efecto mediante la modulación de la producción de ROS y de citoquinas en GAs y en macrófagos. Además, en macrófagos estaría implicada en la polarización hacia fenotipo M2, pudiendo señalizar a través de la ruta cAMP/PKA/CREB.

Finalmente, en el tercer capítulo, determinamos los efectos de la $15dPGJ_2$ y su ruta de señalización en GAs de dorada. Para ello, los GAs fueron estimulados con VaDNA y tratados con la $15dPGJ_2$. A continuación, se analizaron mediante RT-qPCR los niveles de ARNm de los genes que codifican para los distintos PPARs en dorada, así como su modulación tras la estimulación con VaDNA. Los resultados mostraron que dichos genes se expresaban constitutivamente. Sin embargo, la estimulación resultó en un incremento en los niveles de ARNm de *pparg*, exclusivamente. Del mismo modo, la $15dPGJ_2$ produjo un efecto inhibidor de los niveles de transcrito de *pparb* y *pparg* en GAs estimulados.

Para de dilucidar el mecanismo de acción de la $15dPGJ_2$ analizamos el efecto de dicha PG y su análogo biotinilado sobre la producción de ROS y los niveles de transcripción de genes que codifican para citoquinas en GAs estimulados. El análogo biotinilado posee la capacidad de formar aductos de Michael, pero pierde la capacidad de interaccionar con el PPARγ. Los resultados mostraron que la $15dPGJ_2$ inhibía la producción de ROS y los niveles de ARNm de *il1b* y *ptgs*2. Sin embargo, el análogo biotinilado a la misma dosis no ejerció efecto alguno, siendo capaz de inhibir la producción de ROS a dosis 5-10 veces mayores, presentando un efecto dependiente de la concentración.

A continuación, se analizó el efecto del análogo estructural 9,10-dihydro-15d-PGJ_2 (CAY10410), capaz de interaccionar con PPARγ, pero que pierde la capacidad de unirse covalentemente a proteínas. Los resultados mostraron que CAY10410 reducía la producción de ROS reproduciendo el efecto de la $15dPGJ_2$. Del mismo modo, se analizó el efecto de diversos agonistas de PPARγ y se observó que tanto troglitazone como ciglitazone reducían la producción de ROS del mismo modo que la $15dPGJ_2$, sin embargo, rosiglitazone no ejerció efecto alguno. Por otra parte, cuando se trató de bloquear la actividad de la $15dPGJ_2$ mediante cotratamiento con antagonistas de PPARγ se observó que solamente T007097 fue capaz de revertir el efecto de dicha PG, pero no revertía el efecto del agonista troglitazone. Sorprendentemente, el antagonista BADGE resultó tener un efecto inhibidor de la producción de ROS actuando como agonista.

A la vista de estos resultados se procedió al estudio de la activación *in vitro* de los distintos PPARs de dorada. Los resultados mostraron que la 15dPGJ$_2$ activaba la transcripción de PPRE a través de la interacción con PPARα y PPARβ, pero no a través de PPARγ. Del mismo modo, el análogo biotinilado de la 15dPGJ$_2$ también inducía la transcripción a través de PPARα y PPARβ, pero no a través de PPARγ.

Finalmente, analizamos las posibles dianas del análogo biotinilado para la formación de aductos de Michael mediante análisis por HPLC/MS. Los resultados mostraron una interacción del análogo biotinilado con el PPARβ, confirmando de este modo los ensayos de transactivación.

Estos resultados muestran que la 15dPGJ$_2$ posee un papel anti-inflamatorio en la resolución de la inflamación de GAs de dorada, señalizando a través de PPARα y PPARβ, así como por unión covalente a proteínas, como por ejemplo PPARβ.

Así, las conclusiones de la presente tesis son las siguientes:

1. Se han anotado y caracterizado dos ortólogos de la L-PGDS de mamíferos en dorada (PGDS1 y PGDS2). En granulocitos acidófilos estimulados con diversos PAMPs se observa una potente inducción de los niveles de transcripción de *pgds1* que se correlaciona con la cinética de producción de PGD$_2$ en dichas células. Sin embargo, *pgds2* no se expresa en dichas células.
2. Los análogos estables de la PGD$_2$ (16,16-dimetil PGD$_2$ y 11-deoxy-11-metilen-PGD$_2$) y las prostaglandinas de la serie J$_2$ (Δ^{12}-PGJ$_2$ and 15-deoxi-$\Delta^{12,14}$-PGJ$_2$) poseen un efecto anti-inflamatorio en granulocitos acidófilos estimulados inhibiendo la producción de ROS y los niveles de ARNm de *il1b* e *il10*.
3. La concentración de PGE$_2$ en leucocitos de dorada está controlada principalmente por la ruta de síntesis de dicha prostaglandina.
4. La PGE$_2$ produce la polarización de macrófagos hacia fenotipo M2 incrementando los niveles de ARNm de *il10*, *mrc1* y *arg2*.
5. La PGE$_2$ señaliza a través de la ruta cAMP/PKA/CREB en macrófagos de dorada.
6. La PGE$_2$ y su análogo estable, la 16,16-dimetil-PGE$_2$, inhiben la actividad biológica de los granulocitos acidófilos de dorada estimulados con VaDNA, mediante la inhibición de la producción de ROS y la expresión de *il1b*, *il10* e *il6*.
7. La 15-deoxi-$\Delta^{12,14}$-PGJ$_2$ ejerce actividad anti-inflamatoria mediante la inhibición de la producción de ROS y la expresión de *il1b y ptgs2* en granulocitos acidófilos de dorada estimulados con VaDNA.
8. La 15-deoxi-$\Delta^{12,14}$-PGJ$_2$ señaliza a través de interacción con PPARs (PPARα y PPARβ), así como a través de unión covalente a proteínas en la respuesta inmunitaria de dorada.

9. El PPARβ de dorada es una diana con la que interacciona la biotin-15-deoxi-$\Delta^{12,14}$-PGJ_2 en GA de dorada estimulados.

ACKNOWLEDGEMENTS

ANEXXE I: Publications derived from the thesis

1. **Victoria Gómez-Abellán,** Jana Montero, Azucena López-Muñoz, Antonio Figueras, Marta Arizcun, Victoriano Mulero, María P. Sepulcre 2015. Professional phagocytic granulocyte-derived PGD_2 regulates the resolution of inflammation in fish. Developmental and Comparative Immunology 52: 182-191
2. **Victoria Gómez-Abellán**, María P. Sepulcre 2016. The role of prostaglandins in the regulation of fish immunity. Molecular Immunology 69: 139-145
3. Jana Montero**, Victoria Gómez-Abellán,** Marta Arizcun, Victoriano Mulero, María P. Sepulcre 2016. Prostaglandin E_2 promotes M2 polarization of macrophages via a cAMP/CREB signaling pathway and deactivates granulocytes in teleost fish. Fish and Shellfish Immunology 55: 632-641
4. **Victoria Gómez-Abellán,** Ana Belén Pérez-Oliva, Fatma Hermi, Marta Arizcun, Victoriano Mulero, María P. Sepulcre. A role for PPARβ in the anti-inflammatory effect of the cyclopentenone prostaglandin 15-deoxy-Δ12,14-PGJ_2 in professional phagocytes of the teleost gilthead seabream (*in preparation*).

ANEXXE II: Participation in publications during the PhD

1. Sylwia Dominica Tyrkalska, Sergio Candel, Diego Angosto, **Victoria Gómez-Abellán**, Fatima Martín Sánchez, Diana García Moreno, Rubén Zapata Pérez, Alvaro Sánchez Ferrer, María P Sepulcre, Pablo Pelegrín, Victoriano Mulero 2016. Neutrophils mediate Salmonella typhimurium clearance through the GBP4 inflammasome-dependent production of prostaglandins. Nature communications 7:12077
2. Daniel Álvarez-Torres, **Victoria Gómez-Abellán**, Marta Arizcun, Esther García-Rosado, Julia Béjar, María P. Sepulcre 2018. Identification of an interferón-stimulated gene, *isg15*, involved in host immune defense against viral infections in gilthead seabream (*Sparus aurata* L.) Fish Shellfish Immunology 73:220-227

ANEXXE III: Participation in national and international conferences

1. Gómez-Abellán, V., Montero, J., García-Alcázar, A., Meseguer, J., Mulero, V., Sepulcre, M.P. Effect and signaling mechanism of 15deoxi-Prostaglandin J_2 in seabream phagocytes. ISDCI; Murcia, España; 7/2015

2. Gómez-Abellán V., Ruiz-Ballester, M., López-Muñoz, A., García-Alcázar, A., Sepulcre, M.P. Study of the activity of Lipocaline type Prostaglandin D synthase in seabream (Sparus aurata). ISDCI; Murcia, España; 7/2015

3. Montero, J., Gómez-Abellán, V., García-Alcázar, A., Mulero, V., Sepulcre, M.P. Prostaglandin E_2 induces the M2-polarization of macrophages form gilthead seabream (Sparus aurata L.). ISDCI; Murcia, España; 7/2015

4. Hermi, F., Cabas, I., Gómez-González, N.E., Gómez-Abellán, V., García-Ayala, A., Oueslati, R., Mulero V., Sepulcre, M.P. Identification and expression analysis of PGE_2 receptor subtypes in gilthead seabream (Sparus aurata L.). ISDCI; Murcia, España; 7/2015

5. Gómez-Abellán, V., Montero, J., Arizcun, M., Sepulcre, M.P. New insights into the signaling mechanism of prostaglandin D_2 and its cyclopentenone derivates in seabream phagocytes.; Toll2015 TOLL2015; Marbella, España; 10/2015

6. Tyrkalska, S.D., Candel, S., Angosto, D., García-Moreno D., Gómez-Abellán, V., Sepulcre, M.P., Martín-Sánchez, F., Pelegrín, P., Mulero V. Zebrafish guanylate binding protein 4 dictates Salmonella typhimurium clearance by neutrophils through the activation of the inflammasome; TOLL2015; Marbella, España; 10/2015

7. Hermi F., Gómez-Abellán V., Montero J., Sarropoulou E., Oueslati R., Mulero V., Sepulcre MP. Molecular and pylogenetic characterization of PGE_2 receptor subtypes in gilthead seabream (Sparus aurata L.); SEI; Alicante, España; 5/2016

8. Gómez-Abellán V, Mulero V., Sepulcre MP. Evolution of Prostaglandins: have teleost fish an active lipocaline-type prostaglandin D synthase?; I Jornadas del Instituto Murciano de Investigación Biosanitaria; Murcia, España; 11/ 2016

9. Gómez-Abellán V., Pérez-Oliva Ana B., Mulero V., Sepulcre MP. Evolutionarly conserved role of 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 in the regulation of innate immunity across vertebrates.; SEI; Zaragoza, España; 5/ 2017

10. Gómez-Abellán V., Pérez-Oliva Ana B., Mulero V., Sepulcre MP. 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 exerts anti-inflammatory effects through PPARγ signaling and covalent-binding to proteins in seabream (Sparus aurata L.) phagocytes. II Jornadas del Instituto Murciano de Investigación Biosanitaria; Murcia, España; 11/ 2017

11. Álvarez-Torres, D., Gómez-Abellán, V., Montero, J., García Alcázar, A., Carmen Alonso, M., Béjar, J., Sepulcre, M.P. A new role for 15dPGJ2 and $\Delta^{12}PGJ_2$ prostaglandins in type I interferon defense against viral infections in gilthead seabream. 10th International Symposium on Viruses of Lower Vertebrates; Budapest, Hungary; 6/ 2017

12. Álvarez-Torres, D., Gómez-Abellán, V., García Alcázar, A., García Rosado, E., Béjar, J., Sepulcre, M.P.. Identification of an interferón-stimulated gene 15 involved in host immune defense against viral infections in gilthead seabream. 10th International Symposium on Viruses of Lower Vertebrates; Budapest, Hungary; 6/ 2017.

13. Solís-Murgas, L.D., Martínez Navarro, F.J., Gómez-Abellán, V., Martínez, P.J., Junqueira Machado, G., Martins Di Chiacchio, I., Mulero, V. Can Bee Pollen be used as an alternative to live feeds in diets of Danio rerio? ICGEV-LAZEN, Cuernavaca, Mexico; 5/2018.

ANEXXE IV: Research stay in other laboratories during my PhD

Host institution: Department of Agricultural, Food and Nutritional Science, University of Alberta, Canadá, Edmonton, Alberta, Canadá.

Responsible person in the host: Daniel R. Barreda

Stay period: 2nd May 2016 – 2nd Agost 2016