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

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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 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 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 prostaglandin-endoperoxide synthase: the constitutively expressed isoform, PTGS1 (COX1), or the inducible isoform, PTGS2 (COX2) [73]. PGH_2 is converted into PGE_2 through the action of the enzyme PGE synthase (PGES) [52]. Several isoforms of PGES have been described in mammals: the cytosolic (cPGES) and the membrane-associated or microsomal (mPGES) PGES [52]. The latter is highly inducible and displays functional coupling with upstream COX-2 enzymes in cells [52]. 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 [36]. The suppression of 15-HPGD activity

Abbreviations: AGs, acidophilic granulocytes; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; CREB, cAMP response element-binding protein; HPGD, 15 hydroxy-prostaglandin-dehydrogenase; HK, head kidney; IL, interleukin; MACS, magnetic-activated cell sorting; mrc1, mannose receptor c-type 1; PAMPs, pathogen associated molecular patterns; PE, peritoneal exudate; PG, prostaglandin; PGDS, prostaglandin D synthase; PGES, prostaglandin E synthase; PTGS, prostaglandin-endoperoxide synthase; ROS, reactive oxygen species; VaDNA, genomic DNA from *Vibrio anguillarum*.

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has been described in PGE_2-rich and immunosuppressive environments [4].

PGE_2 has a dual role modulating multiple aspects of inflammation in a context-dependent manner, showing proinflammatory and anti-inflammatory activities [36]. 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 [31,36,37]. 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 [47,48,72]. Macrophages are an important source of PGE_2, which acts in an autocrine or paracrine fashion [23]. For example, PGE_2 inhibits the bactericidal properties of macrophages, including phagocytosis [6,36] or the production of reactive oxygen species (ROS) [71]. It also intercedes in the balance and type of immunoregulatory cytokines such as IL-10 or IL-17 [29,31,40], influencing, among other things, the macrophage phenotype [44,48,59,61]. 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 [2,24,27,58,74]. In a similar way to macrophages, PGE_2 exerts inhibitory effects on neutrophil functions such as superoxide anion production [22,25,66,78], chemotaxis [5], the release of cytotoxic enzymes [26] and aggregation [80].

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 [1,11,28,31,36]. 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 [59]. 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 [59].

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 [19]. 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 [64], to up-regulate the expression of IL-10, IL-6 and COX-2, and down-regulate IFNγ, TNFα [7] and MHCII expression [65]. In addition, PGE_2 has been shown to modulate ROS production in head kidney leukocytes from sticklebacks [41] and turbot [55]. The very few studies conducted in head kidney macrophages have been performed in trout, where PGE_2 potentiates the phagocytic response [39] and inhibits ROS production [54]. 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 dislocation 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 [12]. 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 [12]. 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 [60]. Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) [69], 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 [60]. Head kidney macrophage monolayers were then obtained after overnight culture of $G7^-$ fractions in serum-free medium as described earlier [60].

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) [56] 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). The concentrations of PAMPs and stimulation times used have been found to be optimal for the *in vitro* activation of seabream AGs and macrophages [67,68]. 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 [10].