Fish & Shellfish Immunology 42 (2015) 316–324

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Prostaglandin E receptor 4 (PTGER4) involved in host protection against immune challenge in oyster, *Crassostrea hongkongensis*

Fufa Qu [a, b, 1], Zhiming Xiang [a, 1], Fuxuan Wang [a, b], Lin Qi [c], Fengjiao Xu [a, b], Shu Xiao [a], Ziniu Yu [a, *]

[a] Key Laboratory of Marine Bio-resource Sustainable Utilization, Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China
[b] University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China
[c] School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China

ARTICLE INFO

Article history:
Received 21 September 2014
Received in revised form
16 November 2014
Accepted 17 November 2014
Available online 25 November 2014

Keywords:
Crassostrea hongkongensis
Prostaglandin E receptor 4 (PTGER4)
NF-κB pathway
Innate immunity

ABSTRACT

Prostaglandin E receptor 4 (PTGER4) is an essential receptor that can detect various physiological and pathological stimuli and has been implicated in a wide variety of biological processes, including the regulation of immune responses, cytokine production, and apoptosis. In this report, the first mollusk *PTGER4*, referred to as *ChPTGER4*, was cloned and characterized from the Hong Kong oyster *Crassostrea hongkongensis*. Its full-length cDNA is 1734 bp in length, including 5′- and 3′-untranslated region (UTRs) of 354 bp and 306 bp, respectively, and an open reading frame (ORF) of 1074 bp. *Ch*PTGER4 comprises 357 amino acids and shares significant homology with its vertebrate homologs. The results of phylogenetic analysis revealed that *Ch*PTGER4 clusters with PTGER4 from the Pacific oyster. In addition, quantitative real-time PCR analysis revealed that *ChPTGER4* was constitutively expressed in all tissues examined and that its expression was significantly up-regulated in hemocytes and gills following challenge by pathogens (*Vibrio alginolyticus*, *Staphylococcus haemolyticus* and *Saccharomyces cerevisiae*) and pathogen-associated molecular patterns (PAMPs: lipopolysaccharide (LPS) and peptidoglycan (PGN). Moreover, fluorescence microscopy analysis revealed that *Ch*PTGER4 localized to the membrane, and its overexpression significantly enhanced NF-κB reporter gene activation in the HEK293T cell line. In summary, this study provides the first experimental evidence of a functional PTGER4 in mollusks, which suggests its involvement in the innate immune response in oyster.

1. Introduction

Prostanoids are a group of lipid mediators that include prostaglandins (PGs) and thromboxanes (TXs). These mediators are synthesized from arachidonic acid via the cyclooxygenase (COX) pathway in a variety of tissues and cells after various physiological and pathological stimuli [1,2]. Upon production, prostanoids are rapidly released from cells and act as local hormones in the vicinity of their production site to maintain whole-body homeostasis [2,3]. Prostaglandin E2 (PGE2), one of the best known and most well-studied prostanoids, is a proinflammatory mediator that is ubiquitously expressed [4,5] and plays important roles in mediating many inflammatory responses in mammals [6]. The biological actions of PGE2 are mediated by four distinct prostanoid E receptors (EP), termed PTGER1-4 (also known as EP1-4), all of which have seven transmembrane-spanning domains [7,8] and are coupled to a variety of intracellular signal transduction pathways including those that induce increases in cAMP (PTGER2 and PTGER4), decreases in cAMP (PTGER3) and of Ca^{2+} mobilization (PTGER1) [8,9]. Among the PTGERs, PTGER4 is the most abundantly expressed EP subtype in a variety of immune cell types, and its functional responses have been reported in B- and T-lymphocytes, eosinophils, monocytes and macrophages [10,11]. It was reported that the PTGER4 has several sites, including S103, T168, Y186, F191, L195, S285, and D311, which were identified as being essential of the interaction of PGE2 [12]. Activation of PTGER4 by PGE2 has been implicated in a wide variety of biological processes, including the regulation of immune responses and cytokine production [13,14].

PGE2 has been shown to exert anti-inflammatory effects via interactions with different receptor subtypes in mammals, and

* Corresponding author. Tel./fax: +86 20 8910 2507.
E-mail address: carlzyu@scsio.ac.cn (Z. Yu).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.fsi.2014.11.023
1050-4648/

PTGER4 has been demonstrated to be the key mediator of the anti-inflammatory effects of PGE2 [15,16]. It was shown that PGE2-PTGER4 signaling in mammalian macrophages suppressed the stimulus-induced expression of certain proinflammatory genes, including tumor necrosis factor (TNF)-α, interferon (IFN)-β and macrophage inflammatory protein (MIP)-1β [14,17]. Reportedly, in the setting of LPS stimulation, PGE2 signaling via PTGER4 decreased the levels of interleukin-1beta (IL-1β) [18] and inducible nitric oxide synthase (iNOS) [19] in microglia. Additionally, over-expression of PTGER4, as well as PTGER2, resulted in a significant reduction in the LPS-induced mRNA expression of pro-inflammatory chemokine monocyte chemotactic protein-1 (MCP-1) in glomerulonephritis [20]. Moreover, the anti-inflammatory effect of PTGER4 was also observed in LPS-treated BV-2 cells in which PTGER4 activation decreased the phosphorylation of IKKα/β subunits and decreased nuclear translocation of the NF-kappa B subunits p65 and p50, ultimately reducing the transcription of proinflammatory genes [21].

Beyond its key role in anti-inflammatory processes, PTGER4 has also been shown to function as a regulator of pro-inflammatory cellular signaling pathways. For example, it is believed that PTGER4 may be involved in the biosynthesis of matrix metalloproteinases (MMPs) from plaque macrophages that then exert pro-inflammatory effects in the late phase of atherosclerosis [22,23]. A previous study in RAW 264.7 macrophages demonstrated that activation of PTGER4 and PTGER2 by peptidoglycan (PGN) stimulation resulted in increased cAMP levels and activation of protein kinase A (PKA), which in turn increased NF-κB activation and finally induced IL-6 production [24]. Recently, it was reported that IL-8 is an important mediator of the acute host inflammatory response and is produced in response to PGE2 via the activation of a cAMP-dependent mechanism that is mediated exclusively by activation of PTGER4 in colonic inflammation [25].

In recent years, PTGER4 has been attracting significant attention due to its crucial roles in immunity, and its homologs have been identified in various vertebrates, such as *Homo sapiens* [26], *Mus musculus* [27], *Gallus gallus* [28], *Danio rerio* [29]and *Salmo salar* [30]. Many putative PTGER4 have been found in invertebrates, including *Crassostrea gigas* (EKC40150.1), *Aplysia californica* (XP_005095886.1) and *Camponotus floridanus* (EFN71431.1) [31], and subsequently reported in the NCBI database. However, to the best of our knowledge, no experimental evidence of a functional PTGER4 in invertebrates has been provided until now. Therefore, isolation and functional research on the oyster PTGER4 may contribute to a better understanding of its immune function in invertebrates and provide new insight into the immune defense mechanism of oysters. With this in mind, we have cloned a mollusk PTGER4 homolog from *Crassostrea hongkongensis* (designated *ChPTGER4*) and investigated its expression levels in both hemocytes and gills in response to challenge with pathogens and PAMPs. In addition, the subcellular localization and functional role of *Ch*PTGER4 in the NF-κB signaling pathway were also analyzed in HeLa and HEK293T cells, respectively. Taken together, these data contribute to clarifying the possible biological functions of PTGER4 in mollusks.

2. Materials and methods

2.1. Animals, tissue collection and immune challenge

Healthy *C. hongkongensis* individuals, averaging 100 mm in shell height, were collected from Zhanjiang, Guangdong province, China and kept in aerated seawater (salinity, 20‰) at 25 °C. The animals were fed with 0.8% *Tetraselmis suecica* and *Isochrysis galbana* for a week before processing. The tissue distribution experiment was performed according to our previous work [32].

For the pathogen challenge, 200 oysters were randomly divided into 4 groups and placed in 4 tanks for challenge: the *Vibrio alginolyticus* group, *Staphylococcus haemolyticus* group, *Saccharomyces cerevisiae* group and the control group. Individuals in the pathogen challenge groups were challenged by injecting 100 μL of *S. haemolyticus*, *V. alginolyticus* or *S. cerevisiae* (1.0×10^9 cells per liter of PBS) into the adductor muscles, and the individuals in the control groups were injected with an equal volume of PBS (0.14 M sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogenphosphate dodecahydrate, 1.5 mM potassium phosphate monobasic, pH 7.4). After treatment, the oysters were returned to water tanks, and 5 individuals were randomly sampled at 0, 3, 6, 12, 24, 48, and 72 h post-injection. The gills and hemocytes from both challenged and control groups were collected for total RNA extraction.

For the PAMP challenge, hemocyte monolayers were prepared as described in previous reports [33] and challenged as follows: the experimental cultures were incubated with 10 μg/mL LPS (Invivogen) or 10 μg/mL PGN (Invivogen). Control cultures were incubated with an equal volume of PBS. Hemocytes from three replicates were harvested at different time points (0, 1, 3, 6 and 12 h after challenge) for RNA extraction.

2.2. Cloning the full-length cDNA of ChPTGER4 and sequence analysis

One 850 bp EST sequence showing high similarity with invertebrate PTGER4 was obtained from a *C. hongkongensis* hemocyte EST library by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the identified EST sequence, gene-specific primers (Table 1) were designed to amplify the full sequence of *ChPTGER4* using the rapid amplification of cDNA ends (RACE) approach. The 5′-Full Race Kit and 3′-Full RACE Core Set Ver.2.0 (TaKaRa, Japan) were employed to construct the 5′ and 3′ RACE cDNA libraries, respectively, using mixed RNAs from hemocytes and gills of *C. hongkongensis*. PCR amplification to clone the 5′ and 3'ends of *ChPTGER4* was carried out using the gene-specific primers *ChPTGER4*-R1/*ChPTGER4*-R2 and *ChPTGER4*-F1/*ChPTGER4*-F2 (Table 1), respectively. The PCR products were separated by 1.2% agarose gel/TAE electrophoresis and then purified with a TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Japan). After purification, the DNA fragments were ligated into the pMD18-T vector (TaKaRa) and transformed into competent *E. coli* DH5α cells. Randomly selected clones were sequenced on a 3730 Applied

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′–3′)	Comment
Takara5P	CATGGCTACATGCTGACAGCCTA	5′Adaptor
Takara5NP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
Takara3P	TACCGTCGTTCCACTAGTGATTT	3′Adaptor
Takara3NP	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
*Ch*PTGER4-R1	AAAGTCGTATGTAAAGTCCGTGGCA	5′RACE
*Ch*PTGER4-R2	CCAGAGGAGTTCGTTTCCGTGTCAT	
*Ch*PTGER4-F1	ATTCTGTTGACGACGATTGGGCTAA	3′RACE
*Ch*PTGER4-F2	CCAGAATCTACTCCAAACGAGGTCA	
*Ch*PTGER4-F3	GATTTCTTCGGCGATGATTGTGT	qPCR of
*Ch*PTGER4-R3	GAGCTGATCCTGTCCATTGTTGTG	*Ch*PTGER4
GAPDH-F	GGATTGGCGTGGTGGTAGAG	qPCR of GAPDH
GAPDH-R	GTATGATGCCCCTTTGTTGAGTC	
*Ch*PTGER4-F4	AAAAAGCTTCCATGGATGACACGGAA	*Ch*PTGER4-His
*Ch*PTGER4-R4	GGGCTCGAGAAGTTTGTACAAAACAGTC	
*Ch*PTGER4-F5	AAACTCGAGATGGATGACACGGAAA	*Ch*PTGER4-GFP
*Ch*PTGER4-R5	TCCAAGCTTAAGTTTGTACAAAACAGTCG	