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Expression and role of gap junction protein connexin43 in immune challenge-induced extracellular ATP release in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Connexin43 (Cx43) is the best characterized gap junction protein that allows the direct exchange of signaling molecules during cell-to-cell communications. The immunological functions and ATP permeable properties of Cx43 have been insensitively examined in mammals. The similar biological significance of Cx43 in lower vertebrates, however, is not yet understood. In the present study we identified and characterized a *Cx43* ortholog (termed *PoCx43*) from Japanese flounder (*Paralichthys olivaceus*) and investigated its role in immune challenge-induced extracellular ATP release. *PoCx43* mRNA transcripts are widely distributed in all tested normal tissues and cells with predominant expression in the brain, and are significantly up-regulated by LPS, poly(I:C) and zymosan challenges and *Edwardsiella tarda* infections as well, suggesting that *PoCx43* expression was modulated by the inflammatory stresses. In addition, cyclic AMP (cAMP), an essential second messenger, also plays an important role in regulating *PoCx43* gene expression, by which the PoCx43-mediated gap junctional communication may be regulated. Furthermore, overexpression of PoCx43 in Japanese flounder FG-9307 cells significantly potentiates the LPS- and poly(I:C)-induced extracellular ATP release and this enhanced ATP release was attenuated by pre-incubation with Cx43 inhibitor carbenoxolone. In a complementary experiment, down-regulation of PoCx43 endogenous expression in FG-9307 cells with small interfering RNA also significantly reduced the PAMP-induced extracellular ATP release, suggesting that PoCx43 is an important ATP release conduit under the immune challenge conditions. Finally, we showed that extracellular ATP stimulation led to an increased *PoCx43* expression which probably provides a feedback mechanism in regulating *PoCx43* expression at the transcriptional level. These findings suggest that PoCx43 is an inducible immune response gene and an important conduit for immune challenge-induced extracellular ATP release in fish.

1. Introduction

Cellular ATP can be released into the extracellular milieu by a number of different cell types under various physiological and pathophysiological conditions. The released ATP functioned as a potent extracellular signaling molecule can modulate a diversity of cell and organ functions *via* autocrine and/or paracrine signaling cascades [1,2]. In immune system, extracellular ATP is an important endogenous danger-associated molecular pattern that can activate innate immune immunity in several different types of immune cells such as dendritic cells [3] and T cells through action on the cell surface-expressed P2 receptors [4,5].

ATP release can occur through lytic and non-lytic pathways. Several cellular mechanisms have been proposed for the non-lytic ATP release, including 1) vesicular exocytosis that usually occurs in synaptic nerve cells [6], 2) plasma or mitochondrial membrane–associated ATP synthase such as F1Fo ATPase [7], 3) ATP-binding cassette transporters including the cystic fibrosis transmembrane conductance regulator [8] and the multidrug resistance

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gene product mdr (also known as P-glycoprotein) [9], and 4) ion channels such as connexin (Cx) hemichannels, pannexin channels, maxi-anion channels [10,11], volume-regulated anion channels [12], and P2X7 receptor channels [13]. The mechanisms for non-lytic ATP release in response to inflammatory stress in lower vertebrates, however, remain incompletely understood.

Our previous investigations revealed that multiple ATP-gated P2X receptors are expressed in a variety of Japanese flounder *Paralichthys olivaceus* immune tissues and cells [14–16] and extracellular ATP signaling is engaged in NLRC inflammasome-mediated innate immune response in the Japanese flounder [17,18]. These observations addressed the importance of extracellular ATP-mediated signaling in fish innate immunity. We have also showed that the release of cytoplasmic ATP into the extracellular space was elicited in the Japanese flounder head kidney cells upon immune stimulations by pathogen-associated molecular pattern ligands such as LPS and poly(I:C). Importantly, this extracellular ATP release could be inhibited by carbenoxolone (CBX) [19], a gap junction protein blocker, suggesting that pannexin and/or Cx is involved in this process. We have previously identified that pannexin1 hemichannel is an important candidate for extracellular ATP release in the Japanese flounder through forming a permeable pore to allow ATP across the plasma membrane [19] and now focus on the role of the gap junction Cx proteins. There are more than 20 Cx members that have been identified in mammalian cells [20]. Several Cx members have been reported to form hemichannels capable of releasing ATP and the most abundant species Cx43 in immune cells has been extensively studied in this regard [2]. For example, Cx43-mediated ATP release have been identified in neutrophils [21] and astrocytes [22].

In addition to serve as a conduit for extracellular ATP release, Cx43 also plays important roles in the immune system [23,24]. Cx43 has been suggested a key role in pathogen invading, survival and replication and therefore has been proposed as a novel therapeutic target to prevent and treat infectious diseases [24]. It has been evidenced that Cx43 hemichannel signaling plays an important role in the initiation of early innate immune responses in endothelial cells [25]. A variety of studies also confirmed that Cx43 is required for effective activation of dendritic cells [26], NK cells [27] and T cells [28]. In addition, Cx43 was discovered to play an important role in neutrophils recruitment [29], macrophage phagocytosis and the regulation of host response to microbial infection [30].

Even through the role of Cx43 for ATP release and its immune significance have been widely recognized in mammals, the similar functions in fish are still lacking. In the present study we isolated a *Cx43* cDNA ortholog (termed *PoCx43*) from Japanese flounder *P. olivaceus* and revealed that *PoCx43* is a highly inducible innate immune response gene. Using combined biochemistry and complementary molecular approaches, we also for the first time show that PoCx43 is an important conduit for the immune challenge-induced extracellular ATP release in fish. These findings will facilitate our understandings for the details of extracellular ATP-mediated innate immune signaling in fish.

2. Materials and methods

2.1. Fish maintenance and tissue collection

Japanese flounder *P. olivaceus* were obtained from a local fish farm in Dagang, Tianjin, China and maintained in an aerated recirculating seawater system in the laboratory at 21 °C for two weeks before experimentations. Fish were clinically examined before experimentation and only healthy animals without any pathological signs were selected for experiments. Individual tissues form Japanese flounders were separated and collected aseptically as described in the previous study [19].

2.2. cDNA preparation and amplification of Japanese flounder PoCx43 cDNA

Total RNA from *P. olivaceus* tissues was purified by TRIzol reagent (Invitrogen), treated with DNase I (amplification grade, Invitrogen) to remove genomic DNA contamination, and then transcribed into cDNAs using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. No PCR products were amplified from the RNA samples in the absence of reverse transcriptase (data not shown), confirming that there is no genomic DNA contamination.

RT-PCR was applied to amplify the internal fragment of the Japanese flounder *PoCx43* cDNA coding sequence using a degenerate primer pair (F1/R1, Table 1) targeted on the conserved region of Cx43 proteins and the cDNA templates synthesized from Japanese flounder spleen tissue. Based on the obtained *PoCx43* coding sequence, gene-specific primers were design to obtain the 5′- and 3′-untranslated regions of the Japanese flounder *PoCx43* cDNA. Briefly, the 3′-terminal end of *PoCx43* cDNA sequence was amplified with a gene-specific forward primer F2 and a degenerate primer R2 (Table 1). Two rounds of nested-PCR amplification were performed to amplify the 5′-terminal end of *PoCx43* cDNA sequence using SMARTer™ RACE amplification kit (Clontech). The first amplification was done using with a forward primer UPM (a mixture of primers UPM-L and UPM-S, Table 1) and a reverse gene-specific primer GSP (Table 1). The PCR products were then diluted 100 times and used for an additional PCR amplification with primers NUP/GSP (Table 1) in a MyCycler™ gradient thermocycler (Bio-Rad). Finally, the full-length *PoCx43* cDNA sequence that contains the entire coding sequence and 5′/3′-untranslated regions was amplified with gene-specific primer pair F3/R3 (Table 1). All PCR products were separated by 1.2% agarose electrophoresis and stained with ethidium bromide. The PCR products with expected molecular size were ligated into pMD-18T vector (TaKaRa). At least three independent colonies from each transformation were selected for DNA sequencing which showed 100% sequence identity. The complete *PoCx43* cDNA sequence was then deposited in GenBank with accession number KU886276.

2.3. Sequence analyses

The sequence similarities and identities of PoCx43 were determined by blast against GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) using BlastX/P algorithms. The conserved protein domain in PoCx43 was analyzed with the ScanProsite tool (http://prosite.expasy.org/prosite.html) and the SMART tool (http://smart.embl-heidelberg.de/). Transmembrane domains and their orientations were predicated by TMHMM program version 2.0 [31] (www.cbs.dtu.dk/services/TMHMM). Multiple sequence alignments were performed using ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis of the PoCx43 protein and its orthologs from other species was carried out using MEGA (Molecular Evolutionary Genetics Analysis) software version 5.0 and phylogenetic tree was created based on the amino acid sequence alignments of the full-length proteins using the neighbor-joining algorithm and tested for reliability using 1000 bootstrap replications.

2.4. Cell preparation, cell culture, DNA construct and transfection

Japanese flounder head kidney primary cells were prepared as