Developmental and Comparative Immunology 73 (2017) 144–155

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Glycogen synthase kinase-3 (GSK3) regulates TNF production and haemocyte phagocytosis in the immune response of Chinese mitten crab *Eriocheir sinensis*

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ARTICLE INFO

Article history:
Received 24 January 2017
Received in revised form
26 March 2017
Accepted 27 March 2017
Available online 29 March 2017

Keywords:
Eriocheir sinensis
Glycogen synthase kinase 3
Innate immunity
TNF-α production
Phagocytosis

ABSTRACT

Glycogen synthase kinase-3 (GSK3) is a serine/threonine protein kinase firstly identified as a regulator of glycogen synthesis. Recently, it has been proved to be a key regulator of the immune reaction. In the present study, a GSK3 homolog gene (designated as EsGSK3) was cloned from Chinese mitten crab, *Eriocheir sinensis*. The open reading frame (ORF) was 1824 bp, which encoded a predicted polypeptide of 607 amino acids. There was a conserved Serine/Threonine Kinase domain and a DNA binding domain found in EsGSK3. Phylogenetic analysis showed that EsGSK3 was firstly clustered with GSK3-β from oriental river prawn *Macrobrachium nipponense* in the invertebrate branch, while GSK3s from vertebrates formed the other distinct branch. *EsGSK3* mRNA transcripts could be detected in all tested tissues of the crab including haepatopancreas, eyestalk, muscle, gonad, haemocytes and haematopoietic tissue with the highest expression level in haepatopancreas. And EsGSK3 protein was mostly detected in the cytoplasm of haemocyte by immunofluorescence analysis. The expression levels of *EsGSK3* mRNA increased significantly at 6 h after *Aeromonas hydrophila* challenge ($p < 0.05$) in comparison with control group, and then gradually decreased to the initial level at 48 h ($p > 0.05$). The mRNA expression of lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor (*EsLITAF*) was also induced by *A. hydrophila* challenge. However, the mRNA expression of *EsLITAF* and TNF-α production was significantly suppressed after EsGSK3 was blocked *in vivo* with specific inhibitor lithium, while the phagocytosis of crab haemocytes was significantly promoted. These results collectively demonstrated that EsGSK3 could regulate the innate immune responses of *E. sinensis* by promoting TNF-α production and inhibiting haemocyte phagocytosis.

1. Introduction

Glycogen synthase kinase 3 (GSK3) was a serine/threonine protein kinase first isolated and characterized from skeletal muscle about thirty years ago (Embi et al., 1980; Rylatt et al., 1980; Woodgett and Cohen, 1984). The existence of the two main isoforms, GSK3-α and GSK3-β, encoded by different genes has been reported in vertebrates, such as mammals, fish, amphibians, reptiles and lizards, except that GSK3-α gene was absent in birds (Woodgett, 1990). The α and β isoforms of GSK3 share over 98% identity within their conserved Serine/Threonine Kinase domain while differ in their N-terminus (Ryves et al., 2002). The difference of molecular mass between two main isoforms are due to glycine-rich N-terminus of α isoform (Wang et al., 2011). GSK3 has shown to be not only a critical enzyme involved in glycogen biosynthesis but also an important interchange of extensive cell signaling pathways involved in a variety of physiological processes (Wang et al., 2011). For example, it has been reported that GSK3 regulates embryogenesis, cell cycle control, cell motility, cell adhesion and

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http://dx.doi.org/10.1016/j.dci.2017.03.022
0145-305X/

differentiation (Doble and Woodgett, 2003; Jope and Roh, 2006; Jope et al., 2007; Kockeritz et al., 2006).

GSK3 can modulate the key aspects of adaptive immunity, specificity and clonal expansion, by directly regulating proliferation and survival or indirectly modifying the repertoire of cytokines production and influencing differentiation and energy, playing a crucial role in adaptive immunity (Beurel et al., 2010). Researchers have found that GSK3 can regulate several T lymphocyte processes (Garcia et al., 2008; Wood et al., 2006). The role of GSK3 in the regulation of antigen-specific $CD8^+$ T cells has been confirmed and antigen-specific stimulation of $CD8^+$ T cells could result in the loss of GSK3 activity (Ohteki et al., 2000). GSK3 also plays a critical role in costimulatory signal pathway of B7-CD28 (Wang et al., 2011). It is demonstrated that active GSK3 could influence T cell survival, proliferation and differentiation, but the phosphorylated target of GSK3 eventually leading to these effects remains unclear.

In recent years, GSK3 has also been recognized as a critical modulator of various components of innate immune system and an up-rising star in treatment of inflammatory diseases. GSK3 could regulate innate immune response by affecting the pro-inflammatory cytokine production (Beurel et al., 2010). For example, GSK3 deficiency C57BL/6 mice reduced the production of interleukin-6 (IL-6), IL-1b, IL-12p40, IFN-γ and tumor necrosis factor (TNF) by TLR-stimulated monocytes by 67–90% (Martin et al., 2005). IFN-γ can increase the production of inflammatory cytokines induced by lipopolysaccharide (LPS), and this increase is dependent on activated GSK3 (Beurel and Jope, 2009; Lin et al., 2008). Moreover, GSK3 could regulate IL-10 production of monocyte (Chan et al., 2009; Hu et al., 2006; Martin et al., 2005). Inhibitor of GSK3 could increase the production of LPS-induced IL-10 and anti-inflammation IL-1β receptor antagonist, by regulating transcription factors and mitogen-activated protein (MAP) kinases, respectively (Rehani et al., 2009). In vertebrates, the immunomodulation of GSK3 is mainly mediated by GSK3-mediated phosphatidylinositol 3-kinase (PI3K) signal pathway (Kawai and Akira, 2007; Kumar et al., 2009a, b), which plays vital roles in cell proliferation, anti-apoptosis (Osaki et al., 2004), as well as signaling cascade of phagocytic receptors (Allen and Aderem, 1996). All the findings suggest that GSK3 might execute important functions in the innate immune system. However, our knowledge about GSK3 in invertebrates is comparatively limited.

GSK3 homologs have been identified in some invertebrates, including nematodes, molluscs and arthropods. Unlike most vertebrates that harbor both *GSK3* α and β genes, a single gene encoding GSK3 is usually reported in invertebrates, such as β isoform in nematodes, α isoform in choanoflagellates and sea squirts (Alon et al., 2011). In the invertebrates, only a small amount of studies have been so far conducted on GSK3, mainly on its role in glycogen metabolism and development. For example, it was reported that the GSK3 from *Rhipicephalus microplus* and GSK3-β from *Crassostrea angulate* are effective enzymes involved in glycogen metabolism which could regulate gametogenic development during reproduction (Logullo et al., 2009; Zeng et al., 2013). GSK3-β from *Drosophila melanogaster* is involved in embryo dorsoventral axis formation (Bobinnec et al., 2006; Willert et al., 1999). Nevertheless, the roles of invertebrate GSK3 in the immune response are rarely mentioned and detailed mechanism of GSK3-mediated PI3K pathway needs further investigation.

The Chinese mitten crab *Eriocheir sinensis* has long been a critical economic species in China (Li et al., 2007). While with the intensification of high-intensity feeding and environmental degradation over the past few decades, various diseases caused by different pathogens have occurred among the cultured *E. sinensis* populations. As invertebrates, crabs rely solely on innate immune response to protect themselves from pathogen invasion (Wang et al., 2016). Study on the role of GSK3 in regulating the innate immune response of crabs will help us better understand the role of invertebrate GSK3 and the innate immune mechanism of crustaceans. The main purposes of this study are (1) to clone the ORF sequence of GSK3 from *E. sinensis* (designated as *EsGSK3*), (2) to explicit its tissue distribution and cellular localization, as well as its temporal expression profile after bacterial stimulation, and (3) to detect the alteration of immune parameters after EsGSK3 inhibition, hopefully to explore the possible role of EsGSK3 in the innate immune response of crab.

2. Materials and methods

2.1. Crabs and bacterial challenge

Chinese mitten crabs *E. sinensis* were collected from a commercial farm of Lianyungang, Jiangsu province. Before experiment, all crabs were kept in aerated tap water at 20 ± 1 °C for 15 days in order to acclimate to the test conditions.

Ninety crabs were prepared for bacterial stimulation experiment and they were allocated into two groups randomly. Crabs of bacterial stimulation group were injected with 20 μL (10^7 CFU ml^{-1}) of *Aeromonas hydrophila* [*A. hydrophila* was grown in Tryptic-Soy-Broth at 28 °C until 0.8–1.0 of OD_{610}, then bacterial cells were diluted in sterilized physiological saline (0.5% salinity)], while crabs in control group received an injection of the same volume of physiological saline. Six crabs were randomly sampled from each group at 0, 6, 12, 24 and 48 h post injection. Haemolymph from two crabs was mixed as a single sample and there were three biological replicates for each group. And three duplicates were performed for each test.

2.2. Sample collection

Haemolymph was collected using syringes from each crab's second last pair of walking legs 1:1 with precooling anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, 10 mM EDTA). Haemocytes were harvested by centrifuging at 800 *g*, 4 °C, 10 min for the subsequent phagocytosis measurement and mRNA expression analysis. In order to determine the expression pattern of *EsGSK3* mRNA transcripts, various tissues (haepatopancreas, gills, intestine, muscle, stomach, brain and haemocytes) of nine untreated crabs were sacrificed for RNA isolation. All the samples were kept in Trizol reagent (Invitrogen, USA), immediately frozen with liquid nitrogen and kept at −80 °C for subsequent extracting of RNA.

2.3. RNA extraction and cDNA synthesis

The crab's total RNA was extracted from haepatopancreas, haemocytes and other tissues by Trizol reagent, according to protocol of manufacturer. The first strand of cDNA was synthesized by using total RNA (treated with DNase I) as template and oligo (dT) - adaptor as primers according to the protocol of manufacturer of Promega M-MLVRT. The process was carried out at 42 °C for 1 h and terminated by heating at 95 °C for 5 min. The cDNA mixture was diluted at 1: 50 and kept at −80 °C.

2.4. Gene cloning and sequence analysis

A homolog gene of GSK3 (designated as *EsGSK3*) was firstly identified from the cDNA library of *E. sinensis* (Gai et al., 2009). Then, the sequence was blasted in spliced genome of *E. sinensis* accomplished by our laboratory (Song et al., 2016) to get the whole sequence. And the full-length ORF of *EsGSK3* was amplified by