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Cellular Localization of MAPK, NF-κB and Nrf2 Signaling Pathways-Related Proteins in Crayfish Hepatopancreas

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ABSTRACT

Crustaceans rely completely on their innate immune system comprised of various cells, molecules and signaling pathways to survive. Although signaling pathways such as MAPK, NF-κB and Nrf2 have been extensively studied in vertebrates, our current understanding of them in crustaceans is still in the infancy. Studies have found that three members, i.e. p38, RelA (p65) and Nrf2 are evolutionarily conserved, but the specific cells responsible for their synthesis and function have not been well characterized in crustaceans. In this work, we applied an immunohistochemical method to investigate their cellular localization and expression in hepatopancreas of red swamp crayfish (*Procambarus clarkii*), a commercially important aquaculture species in China. Using polyclonal antibodies, the strong staining of these proteins in hepatopancreatic cells was observed. The synchronous expression of p38 and its phosphorylated form (p-p38) was mainly present in B- and R- cells, and their MOD values were 0.0055±0.0038 and 0.0046±0.0027, respectively. As its downstream transcription factors, both RelA (p65) and Nrf2 were widely distributed in the cytoplasm of B- and R- cells, but RelA (p65) showed a relatively higher MOD level compared to Nrf2 (0.0097±0.002 versus 0.0039±0.004). All proteins except Nrf2 could be also obviously observed in sinusoids. The information above implied that B- and R- cells could be predominantly responsible for their function. This study will help us better understand immunity mechanisms of crustacean and promote disease control.

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KW, YW and CS designed the experiments. CS performed the experiments. CS and YW conducted the analysis. KW wrote and revised the manuscript.

Key words
Hepatopancreas, Immunohistochemistry, Crayfish, MAPK, Transcription factors

INTRODUCTION

In recent years, crustaceans have increasingly suffered from environmental stress and pathogenic infection in aquaculture, resulting in serious health risks. As invertebrates, they lack adaptive immunity and must depend completely on innate immune mechanisms, composed of various cells, molecules and signaling pathways, to survive. Studies have indicated that Toll, IMD and JAK/STAT pathways play vital role in regulating immune response of shrimp (Li and Xiang, 2013). There is growing evidence that intracellular signaling molecules such as mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB) and NF-E2-related factor 2 (Nrf2) are evolutionarily conserved and exist in aquatic animals, including many crustacean species. These molecules as well as their interaction, which is dependent on the phosphorylated p38MAPK (p-p38), participate in cellular response to environmental stress and immune challenge (Jeong *et al*., 2017; Song, 2019; Wei *et al*., 2019).

In crustaceans, hepatopancreas combines various functions of vertebrate liver and pancreas. It is also recognized as an important immune organ because hepatopancreatic epithelia are major source of some immune molecules, such as hemocyanin, ferritin, lectins and nitric oxide synthases (Rőszer, 2014). However, cellular damage and tissue injury can cause immune suppression and dysfunction and even endanger their survival. In aquaculture, it is an effective measure to enhance the host's immunity through nutritional regulation targeting hepatopancreas (Lobato *et al*., 2013; Lu *et al*., 2019). The expression and distribution of the members of MAPKs, NF-κB and Nrf2 pathways have been investigated in aquatic animals, and hepatopancreas have exhibited higher mRNA levels compared to other tissues (Kong *et al*., 2011; Rőszer, 2014; Peng *et al*., 2015; Yu *et al*., 2017; Wang *et al*., 2018). It will be crucial to determine the cellular localization of these proteins in tissue for better understanding of their key physiological roles. Research strategy based on cross-reactive antibodies to corresponding mammalian molecules is being increasingly adopted to determine the specific cells responsible for their

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physiological function (Châtel *et al*., 2010; Kang *et al*., 2017).

The red swamp crayfish (*Procambarus clarkii*) is not only a commercially important aquaculture species in China but also frequently used as a model organism for studying infection, immunity and environmental toxicology (Wei and Yang, 2016; Zhou *et al*., 2017). In this study, an immunohistochemical (IHC) method was employed to detect the cellular localization and expression of these signaling molecules: p38 and its phosphorylated form (p-p38), RelA (p65) and Nrf2 in hepatopancreas. This will help us deeply understand immunity mechanism of crustacean and promote disease control.

MATERIALS AND METHODS

Crayfish

Procambarus clarkii were collected from Hubei, one of the largest breeding base of China, and kept in indoor glass aquarium (45 cm × 30 cm × 30 cm) with 3–4 cm depth of continuous aerated and dechlorinated tap water (pH 7.2 ± 0.4). The water temperature was maintained at 16 ± 1 °C and the photoperiod was set to 12:12 (L: D). Crayfish were fed with commercially available feed twice daily and acclimatized for 20 days before the experiment. Every day the unconsumed food and feces were removed carefully with a siphon. Water was changed twice a week after aquarium was cleaned thoroughly. Only healthy adult crayfish (without any damage and pathological signs, body length of 9.2 ± 0.4 cm and wet weight of 21.5 ± 4.3 g) in the intermoult stage were selected and used for the experiments. All animal handling procedures followed the Ethics Committee of Scientific Research in Shanxi University, China.

Chemicals

Anti-p38MAPK antibody (cat. no. 14064-1-AP), anti-RelA (p65) antibody (cat. no. 10745-1-AP) and anti-Nrf2 antibody (cat. no. 16396-1-AP) were obtained from Proteintech Group (Wuhan, China. http://www.ptgcn.com/). Phospho-p38MAPK (Thr180/Tyr182) antibody (cat. no. abs131122) was bought from Absin Bioscience Inc. (Shanghai, China. http://www.absin.cn/). All primary antibodies were raised in rabbit, and their specificity on crayfish hepatopancreas proteins has been validated in our previous studies (Song, 2019; Wei *et al*., 2019). Biotin-conjugated goat anti-rabbit IgG from Boster Biological Technology (Wuhan, China) was used as the secondary antibody. The streptavidin-biotin-peroxidase complex and diaminobenzidine (DAB) substrate kit were supplied by Boster Biological Technology (Wuhan, China) and Beijing Zhongshan Jinqiao Biotechnology (Beijing, China), respectively. All other chemicals were of analytical grade unless stated otherwise.

Sampling

Six crayfish randomly selected were anaesthetized on ice for about 20 min, then hepatopancreas were carefully excised. After rinsing with ice-cold phosphate buffer solution (PBS), tissues were immediately fixed for 48h with 4% paraformaldehyde solution in PBS (pH 7.2) at 4 °C. The preserved tissues were dehydrated in increasing ethanol concentrations, cleared with xylene, embedded in paraffin. Then 5 μm-thick sections were cut using microtome (LeicaRM2255, Germany) for subsequent analysis.

Immunohistochemical analysis

To localize these target proteins in tissue sections by immunohistochemical approach, the indirect avidin-biotin-peroxidase staining technique was used as previously described with some modifications (Giriş *et al*., 2007; Kang *et al*., 2017; Wei *et al*., 2019). Paraffin-embedded sections were deparaffinized in xylene and rehydrated through an ethanol series. After rinsing with PBS, the slices were subjected to a heat-induced antigen retrieval protocol (10 min in boiling 0.01mol/L sodium citrate buffer, pH 6.0) and blocked with 5% normal bovine serum in PBS for 30 min at 30 °C. Slides were stained with primary antibodies in 0.01 mol/L PBS-Tween-20 (PBST) (1:450 dilution for anti-p38MAPK antibody, overnight at 4 °C). Then sections were repeatedly rinsed with PBST and incubated with secondary antibody (1:200 dilution) for 20 min at 24.5 °C. Subsequently, the slices were incubated with avidin–biotin–peroxidase complex for 20 min at 25 °C. Sections were stained with DAB, observed under an Olympus BX51 microscope (Olympus, Japan), and the staining reaction was terminated by rinsing as soon as brown staining was visible. Finally, sections were counterstained with hematoxylin, dehydrated in an alcohol gradient, cleared in xylene, then mounted in neutral resin. As negative controls, primary antibodies were replaced with an equivalent amount of 0.01 mol/L PBST. Different staining protocol for other proteins was shown in Table I.

Images were observed under Olympus BX51 microscope. Cytoplasm or nucleus with brown particles were declared as positive. The intensity of staining was classified as weak or almost absent (—), moderate (+) and intense (++), which was evaluated separately by two experienced pathologists (Ogbureke *et al*., 1995). Five randomly selected fields per slide were evaluated semi-quantitatively by mean optical density (MOD, MOD=IOD/Area) using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Results were

presented as mean ± SD from 6 crayfish.

To provide a corresponding histomorphology map, paraffin slides were stained conventionally with hematoxylin and eosin (H and E).

Table I. An immunohistochemical staining protocol for signaling pathway-related proteins.

PA [a]	PA dilution in PBST [a]	IT of SA (°C) [b]	IT of ABC (°C) [b]
anti-p38MAPK	1:450	24.5	25
phospho-p38MAPK	1:250	26	27
anti-RelA (p65)	1:1400	24	25
anti-Nrf2	1:1600	24	25

[a]PA, primary antibody; PBST, PBS-Tween-20; [b]IT, incubation temperature; SA, secondary antibody; ABC, avidin biotin peroxidase complex.

RESULTS AND DISCUSSION

As shown in Figure 1A, hepatopancreas was essentially composed of branched tubules, which were lined with different types of epithelial cells, i.e. B-cells (secretory), R-cells (absorptive), E-cells (embryonic) and F-cells (fibrillar). Connective tissue lay between adjacent tubules and there were sinusoids in it. B-cell (blister-like) contains a single large vacuole, which occupies 80-90 % of the cytoplasm, and may be involved in absorbing nutrients from the lumen and secreting the digestive enzymes. R-cells are the most abundant hepatopancreatic cell type, generally characterized by considerable number of lipid inclusions, and they combine the functions of intestinal absorptive and hepatic parenchymal cells in vertebrates. It has been found that hepatopancreas is capable of producing and secreting a number of immune-related molecules. For example, hemocyanin is principally synthesized in the F-cells, RelA (p65) in B- and R- cells, NOS/NO in epithelial cells and CBS/H_2S in sinusoids (Rőszer, 2014; Sun *et al*., 2015; Wei *et al*., 2019). Although MAPK, NF-κB and Nrf2 pathways have been well studied in vertebrate, their presence and location in crustacean tissue is largely unknown.

Like a vast majority of aquatic animals, molecular information about these proteins is almost complete absent in crayfish due to the lack of abundant genomic data, resulting in their cellular localization more challenging. Based on polyclonal antibodies, our IHC analysis indicated that p38-like, RelA (p65)-like and Nrf2-like immunopositive cells coexisted in epithelial cells of tubules. The synchronous expression of p38 and its phosphorylated form (p-p38) were mainly found in B- and R- cells, and their MOD values were 0.0055±0.0038 and 0.0046±0.0027, respectively. As its downstream transcription factors, both RelA (p65) and Nrf2 were

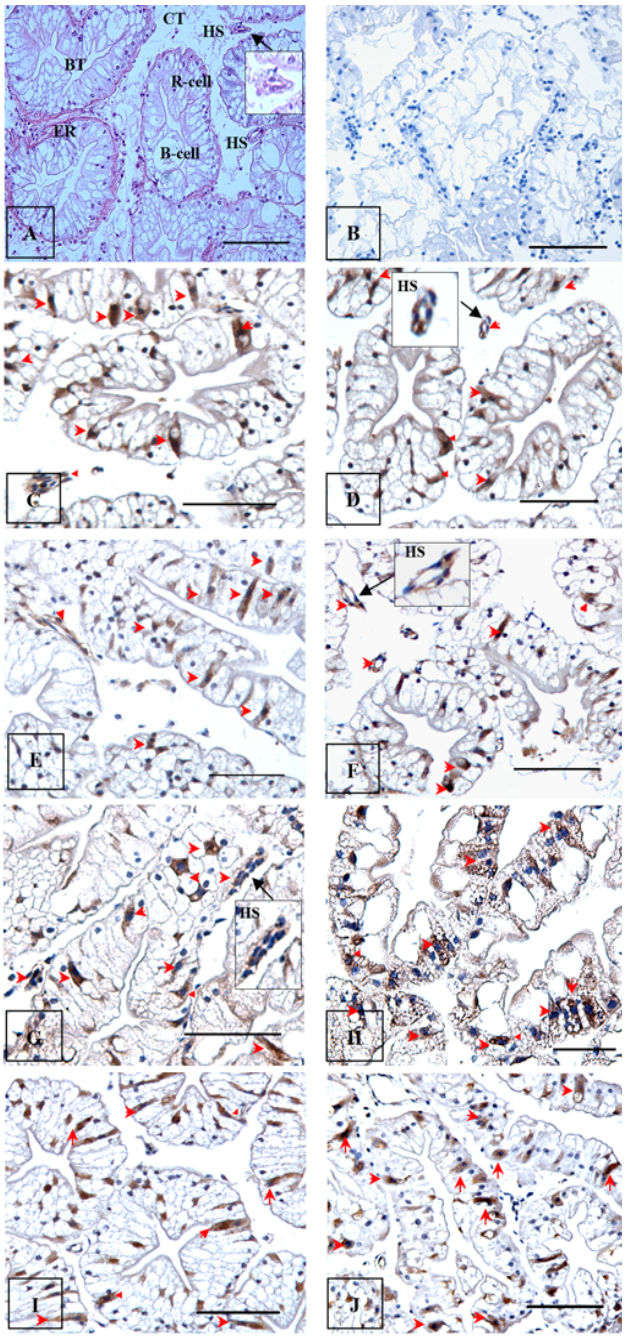

Fig. 1. Localization and distribution of signaling pathway-related proteins in hepatopancreas of red swamp crayfish (*P. clarkii*). A, H and E staining; B, negative control (no primary antibody); C and D, p38MAPK; E and F, p-p38 MAPK; G and H, RelA (p65); I and J, Nrf2. The representative immunopositive cells were indicated with red arrowheads. Square indicated partial enlarged view. B-cell, secretory cell; BT, blind tubule; CT, connective tissue; ER, epithelium; HS, haemolymph sinusoids; R-cell, absorptive cell. 100 ×; scale bars=50 μm.

widely distributed in the cytoplasm of B- and R- cells, but RelA (p65) showed a relatively higher MOD level compared to Nrf2 (0.0097±0.002 versus 0.0039±0.004). Other kinds of epithelia had very weak or almost no staining. All proteins except Nrf2 could be also obviously observed in sinusoids. This information above implied that B- and R- cells could be predominantly responsible for their synthesis and function (Figs. 1B-J, 2, Table II).

Table II. Epithelium showing immunopositive reaction in crayfish hepatopancreas.

Signaling pathway related proteins	Epithelium of tubule [a]			
	B-cell	R-cell	E-cell	F-cell
p38MAPK	++	+	—	—
p-p38MAPK	++	+	—	—
RelA (p65)	++	++	—	—
Nrf2	++	+	—	—

[a]B-cell, secretory cell; R-cell, absorptive cell; E-cell, embryonic cell; F-cell, fibrillar cell; —, very weak or almost no staining; +, moderate staining; ++, intense staining.

In shrimp and crab, p38MAPK gene has been found to mediate cellular response to oxidative stress and pathogenic challenge. Tissue distribution of p38 mRNA has been identified in *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, *Portunus trituberculatus* and *Scylla paramamosain* et al., with the higher expression level in gill, haemocyte, intestine and hepatopancreas (Rőszer, 2014; Peng *et al*., 2015; Yu *et al*., 2017). In mammals, p38 can translocate from cytoplasm to the nucleus in response to various extracellular stimuli following the activation upon dual phosphorylation of its highly conserved Thr-Gly-Tyr (TGY) motif, thus exert multiple roles by targeting specific cells and molecules (Peng *et al*., 2015; Yu *et al*., 2017). In many situations, the upregulation of transcription factors, such as NF-κB and Nrf2, requires the activation of upstream p38 signal, as shown in *Paracyclopina nana*. It is even possible that their potential "cross-talk" is closely associated with the phosphorylation of p38 (Yan *et al*., 2013; Jeong *et al*., 2017). In the present study, we employed an antibody specific to the simultaneous phosphorylation of Thr180 and Tyr182 in the conserved TGY motif.

As a "rapid-acting" primary transcription factor to harmful cellular stimuli, NF-κB exists as homo- or heterodimeric complexes formed in mammals by the Rel homology domain (RHD)-containing proteins NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel. The other three members, viz. Dorsal, Dif and Relish have been identified in *Drosophila melanogaster*. It predominantly comprises p50 and p65 subunits in most cells. Under basal conditions, the p50/p65 complex remains inactive in the cytoplasm associated with inhibitory IκB proteins. Once activated, p65 translocates into the nucleus and modulates the expression of genes crucial for immunity, inflammation and stress responses. There is increasing evidence for the presence of NF-κB homologs in aquatic invertebrate. Their mRNA expression profiles have been detected in hemocytes and hepatopancreas of *Litopenaeus vannamei*, *Carcinoscorpius rotundicauda*, *Chlamys farreri*, *Crassostrea gigas*, *Haliotis diversicolor supertexta* and *Haliotis discus discus* (Kong *et al*., 2011; Wei *et al*., 2019). Because of improvement of staining protocol, RelA (p65) protein was detected not only in epithelia (Wei *et al*., 2019) but also in sinusoids (Fig. 1G and H).

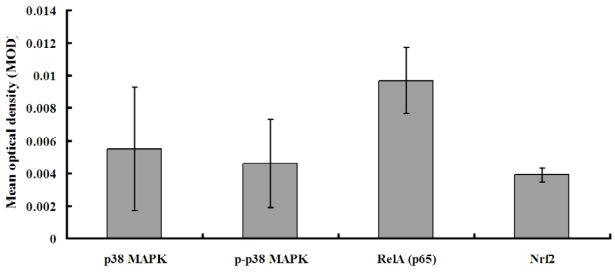

Fig. 2. Tissue distribution of signaling pathway-related proteins in hepatopancreas of red swamp crayfish (*P. clarkii*). Expression levels were quantified as mean optical density (MOD) value of the immunopositive cells. Data were expressed as the mean ± SD (n = 6).

In contrast to NF-κB, little is currently known about the Nrf2 pathway in crustacean, and most of information is mainly obtained from fish and mammalian. It is abundantly expressed in the liver, kidneys and intestine as well as other organs continuously exposed to environment (Giuliani and Regoli, 2014; Wang *et al*., 2018). Under normal conditions, Nrf2 protein is sequestered in the cytoplasm by its inhibitor Keap1, a cytoplasmic protein, and maintained at very low steady-state level. Once stimulated by oxidative stress, Nrf2 dissociates from keap1, translocates into the nucleus, and functions as a "redox switch" to elicit expression of target genes, including detoxifying and defensive enzymes (Lobato *et al*., 2013; Wang *et al*., 2018). Except for the nematode *C. elegans* and the fruitfly *Drosophila melanogaster*, Nrf2 gene has been validated in intestine, gill and digestive gland of just a few of aquatic invertebrate such as *Ruditapes philippinarum* and *Crassostrea gigas* (Danielli *et al*., 2017; Wang *et al*., 2018).

This study further revealed cellular sources of these molecules in crayfish hepatopancreas. We will do in-depth research on their potential roles in immune response.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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