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The role of Nrf2-Keap1 signaling pathway in the antioxidant defense response induced by PAHs in the calm *Ruditapes philippinarum*

Hongdan Wang, Luqing Pan*, Lingjun Si, Jingjing Miao

The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, 266003, China

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ABSTRACT

The NF-E2-related factor 2 (Nrf2) is a master regulator of cellular responses against environmental stresses. In this study we cloned the full-length cDNAs of the RpNrf2 encompassed 2823 bp from the clam *Ruditapes philippinarum (R. philippinarum)*. Sequences alignment and phylogenetic analysis showed that Nrf2 was highly specific in the clams. RpNrf2 expression was detected in gill, digestive gland, mantle and adductor, which the highest transcription level was observed in gill and digestive gland. The gene expressions of RpNrf2, Kelch-like-ECH-associated Protein 1 (Keap1), Cul3-based E3 Ubiquitin Ligase (E3), Glutathione S-transferase (GST-pi), Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) in digestive gland was evaluated by real-time PCR after being exposed to benzo(*a*)pyrene (BaP) (0.25, 1and 4 μg/L) for 15 days, which showed that the expression of Nrf2 significantly increased at day 1 and day 6 after exposure ($p < 0.05$), and there was a negative relationship between the mRNA levels of Nrf2 and Keap1 that indicates the enhancement of Keap1 expression stimulating Nrf2 degradation. RNA interference experiments were conducted to examine the expression profiles of RpNrf2, antioxidant and detoxification genes (GST-pi, Cu/Zn-SOD, CAT and GPx) and Lipid Peroxidase (LPO) level in digestive gland exposed to BaP. The results showed that the mRNA level of Nrf2 was significantly decreased by 63.2%, and the changes of antioxidant and detoxification genes expression were consistent with the Nrf2 gene suggesting that Nrf2 is required for the induction of antioxidant and detoxification genes. Besides, the LPO levels expressed by malondialdehyde (MDA) contents were significant higher compared with the control group at 72 h post dsRNA-Nrf2 injection. In conclusion, our data demonstrated that Keap1 can sense nucleophilic or oxidative stress factors to regulate the Nrf2 signaling pathway together with E3 and Nrf2 signaling pathway plays an important role in modulating gene expression of antioxidant enzymes in bivalve mollusks.

1. Introduction

The detoxification response of PAHs in shellfish is a complex process that involves AhR pathway, phase I, phase II xenobiotic-metabolizing enzymes and phase III transporters [1–6], which could produce a large number of active intermediates and Reactive Oxygen Species (ROS) substances to disturbed the normal physiological function of aquatic organisms [3,4]. To prevent damage caused by ROS, organisms have developed an antioxidant defense system including enzymatic and non-enzymatic to cope with oxidative stress [7]. Numerous mammalian studies have shown that the most several important antioxidant enzymes are SOD which detoxifies superoxide anions, CAT which reduces H_2O_2, GPx which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction, and glutathione reductase (GR) which catalyzes the NADPH-dependent regeneration of glutathione (GSH) from the oxidized form (GSSG) generated by GPx. It has also been widely reported that the intracellular levels of some nonenzymatic antioxidants, such as glutathione, influenced the activity of the enzymatic antioxidants [8]. Considering the important role of antioxidant defense system in preventing the oxidative damage caused by PAHs, studies on the activation mechanism of antioxidant defense system are urgently needed.

Numerous mammalian studies have shown that Nrf2-Keap1 signaling molecules are activated by ROS and play an important role in the transcriptional activation of an array of antioxidant and detoxification genes [9]. The activity of Nrf2 is primarily controlled by Keap1 [10].

Abbreviations: R. philippinarum, *Ruditapes philippinarum*; Nrf2, NF-E2-Related Factor 2; Keap1, Kelch-like-ECH-associated Protein 1; PAHs, Polycyclic Aromatic Hydrocarbons; BaP, Benzo(*a*)pyrene; E3, Cul3-based E3 Ubiquitin Ligase; AhR, Aromatic Hydrocarbon Receptor; EROD, 7-Ethoxyresorufin-O-Deethylase; UGT, Udp Glucuronyl Transferase; SULT, Sulfotransferase; GST, Glutathione S-transferase; SOD, Superoxide Dismutase; CAT, Catalase; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GSH, Glutathione; GSSG, Oxidized Glutathione; ROS, Reactive Oxygen Species; LPO, Lipid Peroxidase; MDA, Malondialdehyde; RNAi, RNA interference; GFP, Green Fluorescent Protein; PBS, Phosphate Buffered Solution

* Corresponding author.
E-mail address: panlq@ouc.edu.cn (L. Pan).

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Under quiescent conditions, Keap1 functions as a substrate adaptor for a Cul3-based E3 ubiquitin ligase (E3), were constantly targeting Nrf2 for ubiquitin-dependent degradation [11]. Under oxidative and electrophilic stresses, Keap1 functions as a sensor for the stress signals lose its activity to interact with Nrf2, leading to reducing degradation of Nrf2 and translocating to the nucleus. Then Nrf2 binds to antioxidant/electrophile-response elements located in the regulatory regions of many defense enzyme genes that enhance cell survival [7] [12]. Furthermore, some scholars believe that the activation and regulation of Nrf2 is based on the mechanism of "hinge and latch model" [13]. This model has been proposed based on the finding in which Keap1 homodimer binds to a single Nrf2 molecule through DLG and ETGE regions two distinct binding sites within the Neh2 domain of Nrf2. Once the electrophilic reagents or oxidants are stimulated, the relatively low affinity DLG region will be dissociated from the DGR region, however, a high affinity for ETGE is still firmly bound to DGR, so Nrf2 cannot be identified and degradated by the ubiquitin ligase. At the same time, the entire process of Keap1 site is still occupied by Nrf2, a new generation of Nrf2 due to the site saturated cannot be combined, so it translocates to the nucleus [13,14]. However, the mechanism Nrf2-Keap1 signaling pathway in bivalves has not been reported. It is of biological significance to verify the mechanism of Nrf2 signaling pathway by detecting the expression changes of Nrf2 signaling pathway related genes under BaP stress.

It is reported that Nrf2 belongs to the cap-n-collar subfamily of basic region–leucine zipper-type transcription factors, and it dimerizes with a small Maf proteins [15,16]. In recent years, over two hundred Nrf2 target genes have been identified through gene expression profiling analysis and chromatin immunoprecipitation analysis, exploiting the Nrf2 gene-knockout mice as a reference, Park (2014) demonstrated that Nrf2 is involved in the differential modulation of phase II antioxidant enzyme (HO-1) expression by interfering RNA (siRNA) experiments, and Nrf2 knockdown reduced the basal level of HO-1 expression [17]. In addition, the Nrf2 target genes encode enzymes which could participate in the detoxification response of PAHs [1–7], including the synthesis and conjugation of glutathione, antioxidant enzymes, drug-metabolizing enzymes, transporters, and pentose phosphate pathway enzymes [18]. It can be speculated that an increase of Nrf2 activity is able to elevate resistance of PAHs stress. There are just a few studies about Nrf2 antioxidant defense induced by metal contaminants in bony fishes [19–28], and the molecular studies about Nrf2 antioxidant defense on mollusk have not been reported. Thus, there may be a close relationship between PAHs and Nrf2, and Nrf2 may play an important role in modulating gene expression of antioxidant enzymes regulated by PAHs in bivalve mollusks. By means of introducing dsRNA molecules into organisms or cells to inhibit the expression of a gene of interest, RNAi has become a very powerful tool for reverse genetics to characterize the function of a novel gene, and it was widely used in many aquatic organisms such as zebrafish [29], *Litopenaeus vannamei* [30–32], *Crassostrea gigas* [33] and so on. However, such investigations about the response of Nrf2 Signaling Pathway induced by PAHs in bivalves have not been reported.

R. philippinarum is a commercially important bivalve that is widely distributed along the coast of China, therefore protection from anthropogenic contaminants is essential for survival and food safety. In this study, we first cloned and characterized the Nrf2 gene from the *R. philippinarum*, and then measured the levels of expression of the Nrf2 gene in the mantle, gill, digestive gland and adductor muscle using quantitative RT-PCR. In order to contribute to a better understanding of the detoxification mechanisms of Nrf2 in bivalves, gene expression of Keap1, SOD and GST in digestive gland was evaluated by real-time PCR after being exposed to benzo(*a*)pyrene (BaP). What's more, we used RNAi technology to explore the Nrf2 target genes to verify the role of Nrf2 in the antioxidant defense signaling pathway in molluscs. The objective of this study is to investigate the molecular mechanism of Nrf2-Keap1 signaling pathway in the antioxidant defense response induced by PAHs in the calm *R. philippinarum*.

2. Materials and methods

2.1. Experimental animals and sample preparation

Healthy calms *R. philippinarum* (shell length 35.57 ± 1.8 mm; shell height 24.47 ± 1.4 mm; mass ± 8.97 1.5 g) were collected from the rope-growing cultures at Red Island (Qingdao, China) and acclimated to laboratory condition in aquarium. Tissues from the digestive glands were collected from six healthy calms, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from the digestive glands using RNAiso Plus reagent according to the manufacture's protocol (TaKaRa, Dalian, China). The integrity of the total RNA was verified by 1.2% agarose gel electrophoresis, and the UV absorbance ratio at 260 nm and280 nm (Ultrospec 2100 pro, Amersham, USA). First-strand cDNA was synthesized from 1 μg of the total RNA using a PrimeScriptTM reverse transcriptase kit (TaKaRa, Dalian, China).

2.2. Cloning the full-length cDNA of RpNrf2

The partial sequences of RpNrf2 were obtained from the transcriptome of the clam *R. philippinarum* established by our laboratory (accession number: SRP034886). The sequences of primers were listed in Table 1 and all of the primers were obtained from Sangon (Shanghai, China). The PCR reaction was performed using a Gradient Mastercycler (Eppendorf, Germany) in a total volume of 25 μL PCR mixture containing 2.5 μL 10 × reaction buffer with 15 mmoL L^{-1} $MgCl_2$, 2 μL of 10 mmoL L^{-1} dNTP mix, 1 μL of 25 μmoL L^{-1} of each primer, 2 μL template cDNA, 16 μL MilliQ water, and 0.5 μL of BioReady rTaq DNA Polymerase (5 U μL^{-1}) (TaKaRa, China). To identify the partial RpNrf2 genes of the splicing transcriptome of the clam *R. philippinarum*, PCR cycles were conducted at 95 °C for 3 min followed by 31 cycles of 94 °C for 30 s, 56.5 °C for 1 min, 72 °C for 3 min, and a final cycle of 72 °C for 7 min. The PCR products were analyzed by electrophoresis in 1.0% agarose gel. The result of PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN BIOTECH, Beijing, China) and ligated into pMD 18-T vector (Takara, Dalian, China). Vectors containing

Table 1
Primer sequences of all genes used in the present study listed.

Primer name	Sequences (5′ → 3′)
Nrf2-F	AGAAGGGGTAAAAATAAAGTGG
Nrf2-R	AAAAACAGCAAAATGAGGAAGC
M13-47	CGCCAGGGTTTTCCCAGTCACGAC
RV-M	GAGCGGATAACAATTTCACACAGG
5′RACE-Nrf2	CGCCTGGCTAACGCAAACCAAAGACT
3′RACE-Nrf2	GCAGAAAGAGAGACAAAAGCAAAAAGAG
RT-Nrf2-F	GCCAACATAGGACAATACAAT
RT-Nrf2-R	GGCTACTCTGACGGGGATA
RT-Keap1-F	CAATACGGTGCCACATAA
RT-Keap1-R	ATTCCTCCCAAGTGTCTGT
RT-E3-F	ATACACCAACCAACGAGCC
RT-E3-R	TCCAGACATTTTACGCACA
RT-SOD-F	CACACTTCCGGCATTACCTCC
RT-SOD-R	TTGATTTCATTGCCCCTCGTC
RT-GST-F	AACAAGGAGACCCACCAGGATTG
RT-GST-R	CGATATGCTTCAGAATGGCGTTA
RT-CAT-F	TTTACGGGAATGAATCTATCG
RT-CAT-R	ACCAAGAGCCTGGAAACC
RT-GPx-F	AGTGTGTCCTTCCTGTTCTC
RT- GPx-R	CTGCTTTATTGTCCTTGTTG
RT-18 S-F	GGACCTCGGTTCTATTTTGTTG
RT-18 S-R	TTTCGCTGTAGTTCGTCTTGC
DsNrf2-F	TAATACGACTCACTATAGGGTGTAGGGAAGGAAGTGTTTG
Nrf2-F	TGTAGGGAAGGAAGTGTTTG
DsNrf2-F	TAATACGACTCACTATAGGGTTGAGTTGCTGATTGTATTG
Nrf2-F	TTGAGTTGCTGATTGTATTG