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三角帆蚌活化蛋白激酶C受体1基因(*HcRACK*1)的克隆及表达分析

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摘要: 为了探究*RACK*1基因在三角帆蚌免疫系统中的调控机制, 采用RACE技术克隆得到三角帆蚌*RACK*1基因(命名为*HcRACK*1)的cDNA全序列, 并对该序列进行分析。结果显示, *HcRACK*1基因全长为1249 bp, 其中开放阅读框957 bp, 编码318个氨基酸。蛋白质结构域分析显示*HcRACK*1含有RACK1家族特有的7个WD40结构域。运用荧光定量PCR技术分析*HcRACK*1在正常组织中及受到嗜水气单胞菌侵染和重金属镉暴露后相关组织表达量的变化。结果显示, *HcRACK*1在各个组织中均有表达, 其中闭壳肌中表达量最高; 嗜水气单胞菌侵染后, *HcRACK*1在血淋巴中的表达量逐渐上升, 24 h时达到峰值, 随后下降; 暴露在100 μg/L浓度的重金属镉中, *HcRACK*1在肝胰腺中的表达量逐渐上升, 在第3天达到峰值; 血淋巴在第2天达到峰值, 随后下降; 鳃中*HcRACK*1的表达量无明显变化。上述结果表明, *HcRACK*1与细菌及镉引起的机体的氧化应激反应有关, 并能参与到机体的免疫反应中。

关键词: 三角帆蚌; *HcRACK*1; 氧化应激; 嗜水气单胞菌; 镉暴露
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活化蛋白激酶C受体1(receptor for activated C kinase 1,RACK1)是一种高度保守的细胞内的受体蛋白[1-5], 涉及到超氧阴离子的产生并在免疫应答中发挥重要作用, 广泛分布于真核生物和原核生物中[6]。它含有色氨酸-天冬氨酸7个重复(WD40), 与G 蛋白 β 亚基高度同源。RACK1最初是从鸡肝脏cDNA文库和人B淋巴母细胞株中克隆而来[7], 是多种蛋白激酶C(protein kinase C, PKC)包括PKC- βII、PKCα和PKCε等的受体[8]。

RACK1不仅能够协调多种重要的细胞活动, 如信号转导、黏附、迁移、凋亡等, 还能在脊椎动物的先天免疫中发挥重要作用[9-13]。有研究发现, 利什曼原虫中, 巴西利什曼原虫(*Leishmania* sp.)[15]和杜氏利什曼原虫(*L. donovani*)[16]等表达的RACK蛋白具有很强的抗原性, 能够诱导和感染机体的免疫系统, 进而产生很强的免疫应答反应[14]。人体免疫功能的老化也与RACK表达量的降低有关[17]。而在小鼠的研究中发现, 其免疫功能下降是由于蛋白激酶C信号转导通路受损, 无法激活RACK, 导致表达量降低[18]。此外PKC锚定蛋白RACK1还能调节神经胶质细胞中雌激素的抗炎活性[19]以及活化免疫细胞, 而且是糖皮质激素诱导的抗炎作用的新型转录目标[20]。由此可见*RACK*基因在免疫调节方面的作用非常显著。然而, 软体动物RACK1是否参与宿主防御反应仍然未知。

三角帆蚌(*Hyriopsis cumingii*)又名三角蚌, 在我国分布广泛, 是我国重要的的淡水育珠经济蚌种[21]。镉是一种有毒重金属, 也是一种典型的环境污染物, 它能引起生物体的氧化应激、代

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谢紊乱、血细胞凋亡等，进而干扰生物体的许多生理过程[22-25]。随着人类活动的增加，更多的镉被排放到水环境中，被底栖软体动物吸收和富集。因此，有必要确定一些生物标志物来评估镉对水生生物的影响。嗜水气单胞菌(*Aeromonas hydrophila*)是水环境中普遍存在的一种细菌，它早已被公认为水产养殖多种物种的病原体。最近，三角帆蚌瘟病对三角帆蚌的养殖造成了重大损失[26]。本实验首次克隆了三角帆蚌*RACK*1基因的 cDNA全长(命名为*HcRACK*1)，利用荧光定量PCR技术检测了该基因在各个组织中的表达情况及嗜水气单胞菌刺激和重金属镉暴露后相关组织表达量的变化情况，以期为进一步探究RACK1家族在三角帆蚌免疫系统中的作用，更好地了解淡水贝类免疫系统和防治病害奠定基础。

1 材料与方法

1.1 实验蚌

本研究所用3龄三角帆蚌采自上海市崇明三角帆蚌养殖基地，体长10~15 cm，采回后刷净蚌壳，置于盛有曝气自来水的水族箱中暂养(水温约15 ℃)，每天投喂小球藻2次。

1.2 实验试剂

RNAiso™ plus、Primescript™ RT-PCR Kit、SYBR premix Ex Taq™、3x-Full RACE Core Set Ver3.0购自宝生物工程(大连)有限公司；$CdCl_2·5H_2O$购自上海展云化工有限公司；BD SMART™RACE cDNA Amplification Kit、Advantage®2 PCR Enzyme System购自BD Biosciences Clontech公司；*E.coli* DH5α，零背景快速连接试剂盒购自天根生化科技(北京)有限公司；引物合成和测序由上海生工股份有限公司完成。

1.3 引物设计

利用引物设计软件Primer Premier 5.0，根据构建的三角帆蚌外套膜cDNA文库中已标注的EST序列分别设计3′RACE 和5′RACE 引物，内参基因为EF-1α[27](表1)。

1.4 样品处理

挑选健康三角帆蚌个体，活体解剖，快速取其闭壳肌、斧足、肝胰腺、血淋巴、鳃、外套膜和性腺7种组织，置于液氮中速冻，–80 °C超低温冰箱保存待用。

表 1 本实验所用的主要引物

Tab. 1 Primers used in the study

引物 primer	引物序列(5′-3′) primer sequence	用途 usage
3′ RACE	TACCGTCGTTCCACTAGTGATTT	3′ RACE (通用引物)
5′ RACE UPM	CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT	5′ RACE (通用引物)
3′ RACE GSP	CGGTGCCAGGGACAAGTCAATC	3′ RACE
5′ RACE GSP	GGACACAGGAGACCC AGTCAGAATG	5′ RACE
RACK1-S	TCTTTTTCGTGGGTGCCG	ORF验证
RACK1-A	GCCATTGATGTTTCATTAGCC	ORF验证
RT-F	TCCTTCCAAGCTTGACACTG	实时荧光定量PCR
RT-R	TGCAATGGTACCCAAGTTGT	实时荧光定量PCR
EF-1α-F	GGAACTTCCCAGGCAGACTGTGC	RT-PCR 内参基因
EF-1α-R	TCAAAACGGGCCGCAGAGAAT	RT-PCR 内参基因

嗜水气单胞菌处理　用1 mL的无菌注射器注射1 mL浓度为$2.5×10^8$ CFU/mL的嗜水气单胞菌重悬液(10%的福尔马林浸泡灭活)，对照组注射等体积的1×PBS，分别于3、6、12、24、36和48 h取其血淋巴和鳃，液氮速冻后，–80 °C超低温冰箱保存待用。

重金属镉处理　根据Kim等[28]和Chio等[29]描述的方法，15只蚌置于100 μg/L的含重金属镉水溶液中5 d，实验期间连续充气，分别在处理时间为1、2、3、4和5 d时进行取样，每次取样随机选取3只蚌，取其肝胰腺、血淋巴和鳃，液氮速冻后，–80 °C超低温冰箱保存待用。

1.5 总RNA的提取

使用RNA裂解液RNAiso Plus，按照说明书提取各个组织总RNA，取3 μL RNA用1%的琼脂糖凝胶电泳检测，并利用ND-1000超微量紫外分光光度计测OD值。

1.6 *HcRACK*1全长的获得

3′末端扩增使用3′-Full RACE Core Set Ver3.0试剂盒，上游特异性引物为3′RACE GSP，下游引物为试剂盒中的3′RACE primer。5′末端扩增使用BD SMARTTM RACE cDNA Amplification Kit与Advantage® 2PCR Enzyme System试剂盒，上游引物为试剂盒中的5′RACE UPM，下游特异

性引物为5′RACE GSP。PCR扩增产物经1.2%的琼脂糖凝胶电泳分离检测，用胶回收试剂盒回收目的片段PCR产物，与pGM-T载体连接构建重组质粒，转化到感受态细胞*E.coli* DH5α，经LB平板(含Amp+、IPTG和X-gal)培养后，筛选重组子进行插入片段检测，对所获得的阳性克隆进行序列测定。

1.7　实时荧光定量PCR

RT-PCR目的基因引物采用GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer)在线设计，内参基因为EF-1α。按照SYBR Premix ExTaqTM (Tli RNaseH Plus) 说明书要求，在Bio-Rad CFX-96实时荧光定量PCR仪上进行，PCR反应体系:SYBR 10 μL，模板1.6 μL，引物F 0.8 μL，引物R 0.8 μL，ddH_2O 6.8 μL，反应体系总体积为20 μL。反应程序采用两步法: 95 °C预变性30 s; 95 °C 5 s，58 °C 30 s，72 °C 50 s，30个循环，65~95 °C获得熔解曲线。其中每个样品的目的基因和内参基因分别进行3次重复，根据数值分析得到各个样品的RQ值即$2^{-\Delta\Delta C_t}$，基因表达水平用mean±SE来表示，并应用SPASS17.0软件采用单因素方差分析(One-Way ANOVA)和最小显著差异法(LSD)比较不同数据组间的差异，P<0.05表示差异显著，P<0.01表示差异极显著。

1.8　*HcRACK*1基因的生物信息学分析

通过ORF Finder(open reading frame finder) (http://www.ncbi.nlm.gov/projects/gorf)确定*HcRACK*1 的ORF及编码的氨基酸序列。用Protparam程序(http://web.expasy.org/protparam/)、Sigal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/)、TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)及ProtScale (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1) 等软件分别预测氨基酸序列的物理参数及信号肽，分析氨基酸跨膜结构及氨基酸序列的疏水区。*HcRACK*1 WD40结构域通过SMART程序预测(http://smart.embl-heidelberg.de)。氨基酸序列上的作用位点通过Motif scan程序进行预测(http://myhits.isb-sib.ch/cgi-bin/motif_scan)。用MEGA6.0软件，构建*HcRACK*1氨基酸序列与其他物种的RACK1氨基酸序列的NJ系统进化树，并用重复1000次的自展(Bootstrap)检验计算各分支的置信值。

2　结果

2.1　*HcRACK*1基因cDNA全长与序列分析

*HcRACK*1的cDNA全长1249 bp (GenBank登录号：KT020849)，其中5′端非翻译区(untranslated region，UTR)103 bp，3′端非翻译区189 bp，有典型的加尾信号(AATAAA)和PolyA尾。该基因开放阅读框为957 bp，编码318个氨基酸，预测分子量大小为35.36 ku，理论等电点为7.09。SMART程序分析*HcRACK*1包括7个WD结构域，Motif Scan程序分析*HcRACK*1含有7个PKC磷酸化位点，4个CK2磷酸化位点，4个N-肉豆蔻基化作用位点。使用SignalP 4.0 预测*HcRACK*1无信号肽，TMHMM 预测*HcRACK*1无跨膜区(图1)。

2.2　HcRACK1与其他物种RACK1的多重比对及系统进化树分析

将推导的HcRACK1氨基酸序列与其他物种的RACK1蛋白进行多重序列比对，发现HcRACK1与来自其他物种包括人的RACK1有着很高的相似度(72.64%~87.9%)。应用Mega 6.0软件按邻接法构建了HcRACK1系统进化树(图2)，结果显示HcRACK1与软体动物RACK1聚为一支(图2)。

2.3　*HcRACK*1 mRNA的组织分布及嗜水气单胞菌刺激下*HcRACK*1在血淋巴和鳃中的瞬时表达

QRT-PCR结果显示*HcRACK*1在各个组织(闭壳肌、斧足、肝胰腺、血淋巴、鳃、外套膜、性腺)中均有表达，以闭壳肌中的表达量最高，其次为斧足和鳃，而在血淋巴中的表达量最低(图3)。三角帆蚌在感染嗜水气单胞菌后，血淋巴中*HcRACK*1的表达水平逐渐升高，在24 h时达到峰值(3.4倍，P<0.01)，随后又恢复到感染前的水平。鳃中*HcRACK*1的表达水平在12 h之前无明显变化，12 h时突然升高(2.3倍，P<0.01)，24 h时仍显著高于对照组(P<0.05)，随后降至感染前水平(图4)。

2.4　重金属镉暴露下*HcRACK*1在肝胰腺、血淋巴和鳃中的瞬时表达

QRT-PCR结果显示肝胰腺中*HcRACK*1的表达量逐渐升高，在第3天达到峰值(5.2倍，P<0.01)，随后恢复到正常水平；血淋巴中的

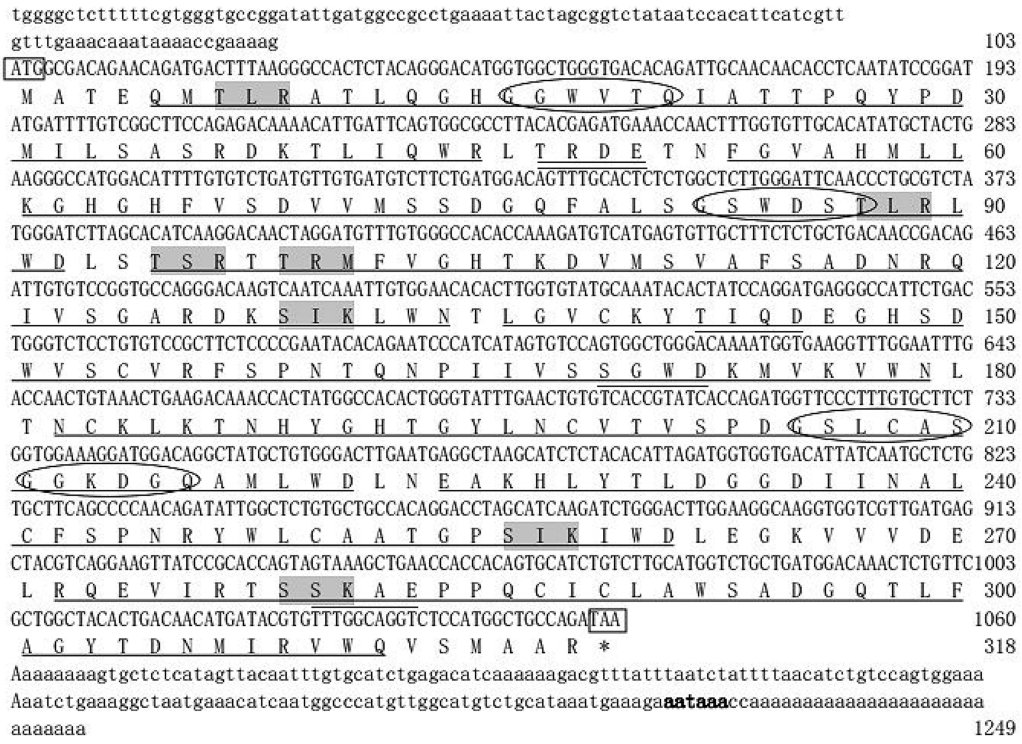

图 1 *HcRACK*1基因核苷酸序列及推导的氨基酸序列

WD40重复用下划线标识，PKC磷酸化位点用灰色阴影标识，CK2磷酸化位点用双划线标识，N-肉豆蔻基化作用位点用椭圆标识，方框内为起始密码子(ATG)和终止密码子(TAA)，加尾信号aataaa加粗标识

Fig. 1 Nucleotide and deduced amino acid sequences of *HcRACK*1

WD40 repeats are underlined, PKC phosphorylation sites are shaded grey with black lettering, CK2 phosphorylation sites are marked with double lines, N-myristoylation sites are marked with ellipse, the start and stop condon is indicated with box, and predicted polyadeny-lation signal is bolded

表 2 不同物种的RACK1的基因登录号及与HcRACK1的相似度

Tab. 2 Genbank accession no. and sequence identities between HcRACK1 from different species

物种名 species	GenBank登录号 acession no.	氨基酸相似度/% amino acid identity
1. 三角帆蚌 *H. cumingii*	KT020849	100
2. 葡萄牙牡蛎 *Crassostrea angulata*	ACU33969.1	87.86
3. 欧洲鸟尾蛤 *Scrobicularia plana*	AFV81452.1	87.74
4. 马氏珠母贝 *Pinctada fucata*	ACJ06767.1	87.07
5. 欧洲牡蛎 *Ostrea edulis*	AFK73701.1	87.7
6. 牛 *Bos taurus*	P63243.3	84.54
7. 人 *Homo sapiens*	AAP36938.1	84.28
8. 虹鳟 *Oncorhynchus mykiss*	NP_001118140.1	84.23
9. 原鸡 *Gallus gallus*	AAW82329.1	83.28
10. 非洲爪蟾 *Xenopus laevis*	AAD42045.1	82.65
11. 家蚕 *Bombyx mori*	ACR19030.1	74.53
12.黑腹果蝇 *Drosophila melanogaster*	AAB72148.1	72.64

*HcRACK*1含量在第1天降低，在2~4 d持续显著上调并在第2天达到峰值(3.3倍，$P<0.01$)，在第5天恢复至正常水平；而鳃中的*HcRACK*1变化幅度较小，显示出较平稳的表达曲线(图5)。

3 讨论

本实验证实该基因与其他物种中的RACK1有着极高的相似度(72.6%~87.9%)，且同样含有RACK家族成员中蛋白保守的7个WD40重复区[30]，以及7个蛋白激酶C磷酸化位点、4个CK2磷酸化位点和4个N-肉豆蔻基化作用位点。系统发育树亦表明该基因与软体动物的RACK1聚为一支。结果证实，*HcRACK*1属于RACK1家族。

*RACK*1基因在脊椎动物和无脊椎动物中对调节体节和肌肉分化起着重要作用，如黄向炜等[31]关于厦门文昌鱼(*Branchiostoma belcheri*)*RACK*1基因在胚胎发育中表达的研究，据此猜测*HcRACK*1可能参与了三角帆蚌肌肉的分化。马氏珠母贝*RACK*1在肝胰腺组织中的表达量最高，推测可能与肝胰腺是一个毒素和细菌累积的中

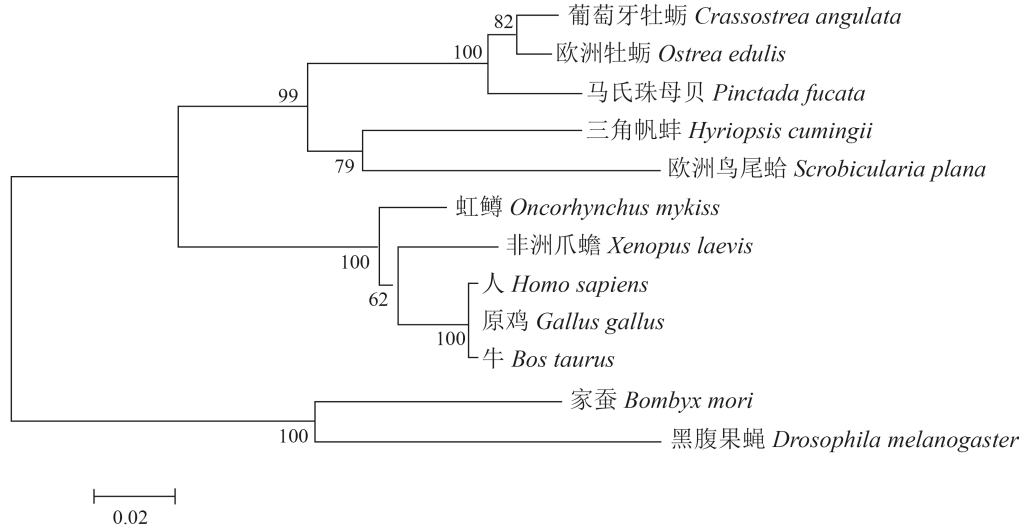

图 2　基于不同物种RACK1氨基酸序列构建的NJ系统发育树

Fig. 2　Phylogenetic tree derived from multiple alignments of RACK1 amino acid sequences from various species

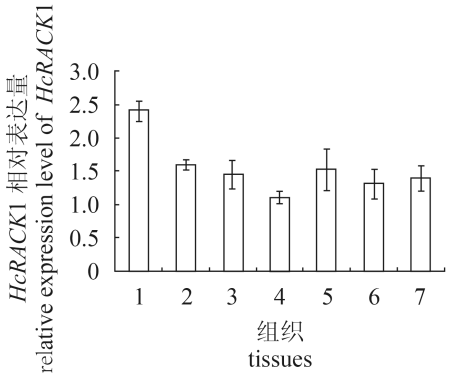

图 3　荧光定量PCR 检测*HcRACK*1 mRNA 在不同组织中的表达量

1. 闭壳肌；2. 斧足；3. 肝胰腺；4. 血淋巴；5. 鳃；6. 外套膜；7. 性腺

Fig. 3　Expression analysis of *HcRACK*1 mRNA in various tissues by qRT-PCR

1. adductor muscle; 2. foot; 3. digestive gland; 4. haemolymph; 5. gill; 6. mantle; 7. gonad

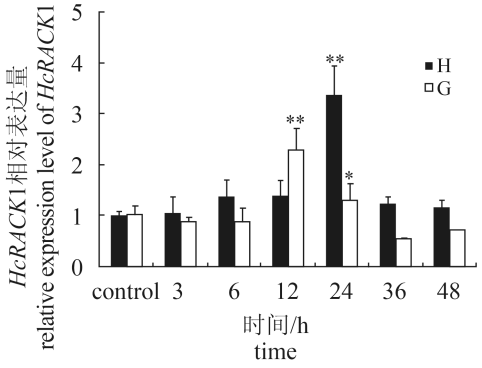

图 4　嗜水气单胞菌刺激下*HcRACK*1基因在三角帆蚌血淋巴(H)和鳃(G)中的相对表达量

Control.0 h; *.与0 h相比较*HcRACK*1基因表达水平存在显著差异(P<0.05); **.与 0 h相比较*HcRACK*1基因表达水平存在极显著差异(P<0.01)

Fig. 4　Temporal expression pattern of *HcRACK*1 mRNA in haemolymph (H) and gill (G) challenged with *A. hydrophila*

Control. 0 h; *. significant difference of *HcRACK*1 gene expression compared with 0 h (P<0.05); **. extremely significant difference of *HcRACK*1 gene expression compared with 0 h (P<0.01)

心，能够诱导机体氧化压力相关基因的表达[13]。大野砂海螂(*Mya arenaria*)*RACK*1在卵巢中的表达量最高，其次为外套膜、精巢和外套膜，但未检测其闭壳肌和肝胰腺中的表达量[32]。猪*RACK*1在胸腺、脑下垂体、脾和肝脏中都高度表达，而在肌肉中未检测到[33]。本实验中，*HcRACK*1在各个组织中广泛表达，其中闭壳肌中的表达量最高。由此看来，*RACK*1的表达模式在不同物种中差异较大。

软体动物马氏珠母贝在感染溶藻弧菌(*Vibrio alginolyticus*)后血淋巴中的*RACK*1含量随着时间呈现U型波动，表现出与氧自由基的产生步调相反的模式[13]。而三角帆蚌在感染嗜水气单胞菌之后，*HcRACK*1在血淋巴中的瞬时表达呈现出逐渐上升随后下降的趋势，但鳃中则是突然上升的表达模式，同时峰值出现早于血淋巴且反应持续时间较长。据此猜测三角帆蚌在注射嗜水气单胞菌后产生活性氧(reactive oxygen species，ROS)，ROS的积累致使机体损伤，然后激发

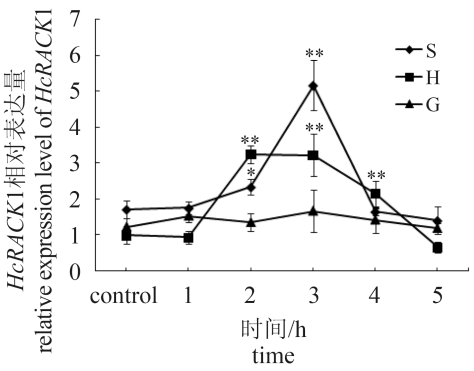

图 5 重金属镉刺激下*HcRACK*1基因在三角帆蚌肝胰腺(S)、血淋巴(H)和鳃(G)中的相对表达量

Fig. 5 Temporal expression pattern of *HcRACK*1 mRNA in digestive gland (S), haemdymph (H) and gill (G) challenged with Cd

*HcRACK*1基因的表达。

重金属镉可以在生物体内富集并通过食物链对人体造成危害。Bouilly等[34]研究发现镉可以影响长牡蛎(*C. gigas*)的生存但对血细胞作用有限。Cheng等[35-36]发现1 mg/L的镉可以诱导提升美国牡蛎(*C. virginia*)的吞噬作用及增加血细胞的数目。肝胰腺和血淋巴中*HcRACK*1的表达量整体上都呈现先上升后下降的趋势，而在鳃中则无明显变化，这种表达模式与暴露在100 μg/L重金属镉中马氏珠母贝的表达模式明显不同。贝类的鳃与水环境接触密切，对水质变化应更为敏感，但RACK1表达量无明显变化，其中的调控机制还需进一步探究。

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Molecular cloning and expression analysis of receptor for activated C kinase 1 (*HcRACK*1) from *Hyriopsis cumingii*

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Abstract: In the present study, full length cDNA sequence of receptor for activated C kinase 1 gene of *Hyriopsis cumingii*(*HcRACK*1) was cloned by using 3'RACE and 5'RACE techniques, and the sequence and structural analysis of the *HcRACK*1 gene was conducted by using bioinformatics method. The results showed that the full-length cDNA of *HcRACK*1 consisted of 1249 bp in length, containing an open reading frame (ORF) of 957 bp encoding 318 amino acids. Analysis of protein domain features showed that the deduced polypeptide contained seven WD40 domains characteristic of RACK1 protein family. The tissue distribution of *HcRACK*1 in unchallenged *H. cumingii* and temporal expression pattern of *HcRACK*1 challenged with bacteria and exposed to 100 μg/L cadmium were analyzed by quantitative real-time PCR (qRT-PCR). The transcript was detected in all tissues tested, and the expression level was the highest in adductor muscle. After challenge with bacteria, expression level of *HcRACK*1 in haemocytes was gradually increased and until 24 h post challenge reached the peak. After exposure to cadmium, its expression level in digestive gland gradually increased and reached the peak on 3 d post exposure, and in haemocytes on 2 d post exposure, and then increased over time. It showed no obvious change in gill. These results suggested that *HcRACK*1 was related to the oxidative stress response caused by bacterial challenge and cadmium exposure, and it also has a potential link with organism's immune response.

Key words: *Hyriopsis cumingii*; *HcRACK*1; oxidative stress; *Aeromonas hydrophila*; cadmium exposure

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