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A house fly TNF ortholog Eiger regulates immune defense via cooperating with Toll and Imd pathways

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ABSTRACT

In mammals, the TNF family is important inflammatory cytokines. Eiger, the invertebrate ortholog of TNF identified firstly in *Drosophila*, has been implicated in immune response with an unknown molecular mechanism. The present work reports a novel *eiger* like gene (*Mdeiger*) from *Musca domestica*. *Mdeiger* was significantly up-regulated upon challenge with either *Escherichia coli* or *Staphylococcus aureus*. Silencing *Mdeiger* led to higher mortalities of larvae post either *E. coli* or *S. aureus* infection, enhanced the expressions of *attacin* and *diptericin*, but blocked the induction of *ceropin* and *muscin*, and inhibited the activation of phenoloxidase following bacterial challenge. Meanwhile, the expression of *dorsal* and *JNK* was inhibited while that of *relish* was enhanced in *Mdeiger*-depleted larvae. We suppose that, by coordinating with the Imd, Toll and JNK pathways, *Mdeiger* be involved in regulating the innate immune response through controlling the capacity of phenoloxidase and the expression of antimicrobial peptide genes synergistically.

1. Introduction

Innate immune system plays a very important role in combating various microbial infections in all animals. Among the immune system, the tumor necrosis factor (TNF) family members and their receptors are important pleiotropic cytokines that can regulate infections, inflammation, autoimmune diseases and tissue homeostasis (Locksley et al., 2001). The first invertebrate ortholog of *TNF* was identified through an ectopic expression screen in *Drosophila* compound eye for genes that cause cell death, which was named *eiger* (EDA-like cell death trigger) (Igaki et al., 2002). As the unique TNF family member found in *Drosophila*, eiger is a type II transmembrane protein, contains a cytoplasmic domain, a transmembrane region and an extracellular domain. The C-terminal TNF homology domain (THD) of eiger, which establish as essential determinants of eiger's activity (Narasimamurthy et al., 2009), shows comparable homology to human TNF family members (Moreno et al., 2002). Like some mammalian TNFs, eiger is able to induce apoptosis. However, unlike its mammalian counterparts, eiger does not require the activity of the caspase-8 homolog DREDD, but completely depends on its ability to activate the JNK pathway and subsequently trigger death (Moreno et al., 2002). Some researches provide evidences that eiger plays a critical role in immune system by transmitting signals. Parisi et al. found that epithelial tumors can trigger a systemic immune response through activation of eiger/TNF signaling, which leads to Toll pathway up-regulation in *Drosophila* adipocytes (Parisi et al., 2014).

Musca domestica (Diptera: Muscidae), the common house fly, develops in vast numbers in animal manure and human excrement where bacteria are abundant, worse than *Drosophila*, yet it seldom exhibits ill effects. Little is known of the impact of environmental and ingested microorganisms on house flies, although they are believed to possess an effective innate immune system to eliminate potential pathogens. Multiple signaling cascades, including the Toll, Imd, JNK and JAK-STAT pathways, can activate components of humoral and cellular responses (Tsakas and Marmaras, 2010). These responses culminate effector mechanisms, including the expression of AMPs that target and kill microorganisms by binding anionic bacterial or fungal membranes leading to disruption and cell death (Yount and Yeaman, 2004). In addition, melanization of hemolymph has a pivotal role in defense against a wide range of pathogens in some dipteran insects. The phenoloxidase (PO) cascade regulates the melanization of hemolymph in insects (Marmaras and Lampropoulou, 2009). Literature suggest that house fly has also become a suitable model organism for studying immune and stress response mechanisms. Therefore we believe it is important to identify and functionally characterize the *Mdeiger* in the house fly system to illustrate the commonalities and differences

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between fruit fly and house fly. In order to further describe the *eiger* molecular immune mechanisms in house fly, we characterize the *Mdeiger* gene from *M. domestica*, examine its mRNA or protein expression in different developmental stages and tissues, in response to infection with bacteria, and study *Mdeiger* RNAi profiles of AMP genes expression and PO activity after bacterial immunostimulation.

2. Materials and methods

2.1. Samples and challenge experiments

The house fly strain was provided by Miss Fengqin He, Institute of Zoology, Chinese Academy of Sciences. Larvae were raised in a climate room at 25 °C, and fed on a medium made of bran (55 g), heat-inactivated yeast (3 g), water (150 ml) and antimycotic nipagin (0.35 g) until pupation. After eclosion, adult flies were fed on water, sugar and milk powder. Flies were maintained at 25 °C under 12 h light/12 h dark cycles (LD12:12) (Codd et al., 2007). Only the larvae with similar size and body weight were used for subsequently experiments. Hemolymph and tissues were collected for RNA extraction according to a previously described method (Gao et al., 2015). Briefly, the anterior tip of the larvae was cut off with a pair of fine scissors and 100 μl hemolymph was pooled from about 30 3rd instar larvae. The fat body, gut and epithelium were excised from the larvae (N = 6) under a binocular microscope (Nikon SMZ1500, Sendai, Japan) and preserved in liquid nitrogen to determine the tissue distribution of the target gene. Each experiment was performed at least 3 times.

The 2nd instar larvae were challenged with Gram-negative bacterium *Escherichia coli* and Gram-positive bacterium *Staphylococcus aureus* for the immune challenge according to the method described previously (Gao et al., 2015). Control larvae injected with phosphate-buffered saline (PBS) were maintained in normal condition. The infected larvae were sampled for RNA or protein extraction at 3, 6, 12, 24, and 48 h post bacterial challenge. Six larvae were randomly tested for each time point, and each experiment was performed at least 3 times.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from entire body or diverse tissues of individuals using RNAiso Reagent (Takara, Dalian, China) according to the manufacturer's instructions and treated with RQ1 RNase-Free DNase (Promega) to remove contaminated DNA. cDNA was synthesized from 2 μg total RNA by M-MLV reverse transcriptase (Promega) following the manufacturer's protocol with an Oligo anchor R primer (Table 1).

2.3. cDNA cloning and bioinformatics analyses

An expressed sequence tag (EST) of suspected *eiger* gene was obtained by searching our transcriptomic database for *M. domestica* (Liu et al., 2012). And accordingly, gene-specific forward primer Mdeiger-F1was designed within 5′ untranslated region (UTR) to clone the 3′ UTR fragment of *eiger* with 3'anchor R (the primer sequences in Table 1). The PCR products were gel-purified and cloned into pMD18-T vector (Takara). After being transformed into the competent cells of *Escherichia coli* DH5α, the positive recombinants were identified through anti-Ampicillin selection and PCR screening. Three of the positive clones were sequenced randomly.

The deduced amino acid sequence was obtained using an ORF finder program (http://ncbi.nlm.nih.gov/gorf/gorf.html). The homology search of the Swissport database was performed using BLASTp algorithm at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org). The protein domains were searched via web CD-search tools, including SMART for Ensembl database (http://smart.embl-heidelberg.de) and Batch for NCBI database (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). The protein sequences in GenBank that can be significantly aligned with Mdeiger's conservative TNF domain were collected to do multiple sequences alignment by ClustalW program. An unrooted phylogenic tree based on the alignments of the TNF homology domains was constructed by the Neighbor-Joining (NJ) algorithm using the MEGA program.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

Tissue distribution and relative expressions of *Mdeiger* at the 1st, 2nd, 3rd instar larvae, pupae and adults were studied by qPCR. Another reaction carried out to determine the mRNA expression profile in the house fly after treatment experiments. The primers Mdeiger-F3 and Mdeiger-R3 were used for amplifying *Mdeiger* fragment with *GAPDH* as internal control for their expression analysis. qPCR was conducted on a LightCycler system (Roche) using the SYBR Green kit from Takara. Expression levels of the target gene were calculated by comparing the cycle threshold value (Ct) to the reference gene *GAPDH*. The relative quantification (comparative method) was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). All samples were normalized to the ΔCt value of a reference gene to obtain a ΔΔCt value (ΔCt target − ΔCt reference). The final relative expression was calculated using the following formula: $F = 2^{-\Delta\Delta Ct}$. The significance at $P < 0.05$ was analyzed using one-way ANOVA.

2.5. RNAi, transcription analysis of AMPs, phenoloxidase activity assay and viability assessment

To investigate the roles of Mdeiger in regulation of the expression of AMP genes, the mRNA from six larvae in each group was isolated and subjected into transcription analysis of AMP genes *attacin* (GenBank: AAR23768), *diptericin* (GenBank: FJ794602), *cecropin* (GenBank: AF416602), and *muscin* (GenBank: KF514663) in *Mdeiger*-depleted larvae. Considering that the Imd and Toll pathways are the major regulators of the immune response in insect, combinatorial knockdown of *Mdeiger/Imd* and *Mdeiger/MyD88* was performed by injection of mixed dsRNA. At 24 h after the dsRNA (dsGFP, dsMdeiger, ds(Mdeiger/Imd) or ds(Mdeiger/MyD88)) injection, mixture of bacterial suspension *E. coli/S. aureus* (1:1, about 10^8 CFU/ml) was injected into the larvae. At 48 h post bacterial challenge, the expression profiles of AMP genes were monitored by qPCR. The activities of Imd, Toll and JNK pathways were measured by detecting the mRNA levels of *relish* (GenBank: XM005178615), *dorsal* (GenBank: XM005182969.2) and *JNK* (GenBank: XM005191342.3) in these RNAi systems post *E. coli* or *S. aureus* challenge. A pair of RNAi assay primers (Mdeiger-F4 & Mdeiger-R4, GFP-F & GFP-R, MyD88-F1 & MyD88-R1, Imd-F1 & Imd-R1), each linked to the T7 promoter, was used to amplify the templates for dsRNA synthesis. DsRNAs were generated using the T7 High Efficiency Transcription Kit (Transgen Biotech) according to the manufacturer's instructions. After purification, the final concentration of dsRNA was adjusted to 2 μg/μl. 20 ng dsRNA was injected into the abdomen of the 2nd instar larvae. At least six individual larvae were injected and the experiments were repeated three times. The dsGFP RNA was used as control. Primer sequences for amplification of AMP genes (*attacin*, *diptericin*, *cecropin* and *muscin*) and *relish*, *dorsal* and *JNK* were listed in Table 1. Meanwhile, total proteins were extracted from Mdeiger-depleted larvae following bacterial challenge for PO activity assay. Oxygenase activity of PO was monitored in a U-721 spectrophotometer at 490 nm by dopachrome formation accompanying the oxidation of the substrate (L-tyrosine or L-3, 4-dihydroxyphenylalanine [L-DOPA]). The reaction medium (3 ml) contained 20 μg PO and 2.0 mM substrate in 50 mM sodium phosphate buffer, pH 6.8. Each assay was carried out in triplicate at a constant temperature of 30 °C, with a 5-min incubation period (Sun et al., 2008). To investigate the effect of Mdeiger on the viability of fly, the survival rates of the *Mdeiger*-depleted larvae were counted following bacterial challenge at 6, 12, 24, 36 h post infection.