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Odorant receptor might be related to sperm DNA integrity in *Apis cerana cerana*

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ABSTRACT

Olfactory receptors (ORs) are important for insects to recognize and discriminate odorants in the environment and are mainly expressed in olfactory and gustatory organs. Little is known about the potential OR functions in non-olfactory tissues. In the present study, we evaluated the possibility of odorant receptors AcerOr1 and AcerOr2 (AcerOr2 is orthologous to the co-receptor) mediating sperm DNA integrity, and the relationship between sperm DNA integrity and semen parameters in *Apis cerana cerana*. Based on previous findings in mammals, we speculated that the Ca^{2+}/calmodulin (CaM)/CaM-dependent protein kinase II (CaMKII) signaling pathway might be involved in the regulation of sperm motility in *A. cerana cerana*. The results showed that both AcerOr1 and AcerOr2 are expressed in the sperms and testis, that components associated with the putative Ca^{2+}/CaM/CaMKII signaling pathway are present in *A. cerana cerana* sperms, and that at least CaM and CaMKII are localized in the sperms and testis. The AcerOr2 agonist VUAA1 significantly improved sperm motility parameters and apoptosis of sperm cells effect DNA integrity, whereas the CaM inhibitor W7 decreased sperm motility parameters and apoptosis of sperm cells, which affects DNA integrity. We also found a positive correlation between sperm DNA integrity and semen quality. These results indirectly as well as directly suggest that OR-mediated sperm responses and the Ca^{2+}/CaM/CaMKII signaling pathway might affect semen quality and might be useful in regulating insect reproduction in future.

1. Introduction

Insect olfactory receptors (ORs) are heteromeric, ligand-gated, seven-transmembrane ion channels that consist of a highly conserved co-receptor subunit (Orco) and an odorant-specific binding subunit (OR). They are mainly expressed on the dendrites of olfactory receptor neurons (ORN) in olfactory and gustatory organs (Franco et al., 2016; Xu et al., 2016; Zhang et al., 2017), and participate in the detection of volatile odors and the conversion of these chemical cues into electrical signals (Wicher et al., 2008; Jones et al., 2012).

The OR/Orco complex forms a functional cation channel allowing K^+ and Ca^{2+} influx into sensory cells upon odorant binding. A conserved calmodulin (CaM)-binding region was found in the Orco intracellular loop2 domain, which indicated that CaM activity might affect the function of Orco (Yap et al., 2000; Mukunda et al., 2014). In mammals, Ca^{2+} plays a critical role in the regulation of sperm functions. For example, in mouse sperm, MOR23 regulates the sperm motility associated with intracellular Ca^{2+} (Fukuda et al., 2004), while inhibition of CaM decreases sperm motility (Ignotz and Suarez, 2005; Schlingmann et al., 2007). CaM-dependent protein

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kinase II (CaMKII), a common downstream target protein of CaM, has been reported to be present in the testis in mammals. The Ca^{2+}/CaM/CaMKII pathway affects and regulates sperm motility in mammals (Schlingmann et al., 2007; Lasko et al., 2012). These results indicate that Ca^{2+}-mediated sperm motility requires CaM.

Several recent studies in *Anopheles gambiae*, *Aedes aegypti*, and *Drosophila melanogaster* provided evidence that Orco is expressed in the sperm, and showed that sperms are activated by an Orco agonist and inhibited by an Orco antagonist (Hansen et al., 2014; Pitts et al., 2014). An intriguing possibility is that Orco mediates the response of spermatozoa to endogenous signaling molecules. To the best of our knowledge, the function of *Apis cerana cerana ORs* (*AcerOrs*) in non-olfactory reproductive tissues has never been investigated. Therefore, this study aimed to explore the possible biological functions of CaM and Orco in the regulation of semen quality and their effect on DNA integrity in the sperms of *A. cerana cerana*; these two factors are critical to the productivity of the honeybee queen, which is limited by the amount of viable sperm that can be stored in its spermatheca for an extended time.

2. Materials and methods

2.1. Chemicals

N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl) thio) acetamide (VUAA1) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl (W7) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Drone rearing, testis and sperm sampling

All *A. cerana cerana* drones (male honeybees) were reared in standard strong honeybee colonies and were periodically fed sucrose syrup. Sexually mature drones were captured at the entrance of a hive when the bees returned from their daily mating flights in the early afternoon. The drones were taken to the laboratory for sample collection. Some of the drone were killed and dissected immediately in the 1% NaCl solution. The prominent fat body surrounding the testis was removed, and the outer membrane of the testis was collected for expression assay. Some of the drones were stimulated by gently pressing the abdomen with two fingers, which resulted in the appearance of the ejaculate at the eversion of the penis. The semen was collected in a sterile Eppendorf pipette. Approximately 0.3–1 μL of ejaculate was collected from each drone and was diluted in 200 μL of 0.9% NaCl solution. Each sample was gently mixed by inversion. Twenty semen samples were collected and analyzed. Sperm viability was assessed by a routine method used for mammalian semen in a sperm cytomorphological study, using the Integrated Semen Analysis System (ISAS® V1, Proiser R + D; Valencia, Spain).

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The transcript levels of *AcerOr* genes were determined by qRT-PCR. Total RNA was isolated from sperms and testis using TRIzol reagent (Takara, Dalian, China), according to manufacturer's protocol. cDNA was synthesized from the total RNA by using a PrimeScript® RT Reagent Kit (TaKaRa), according to manufacturer's instructions. The primers used for *AcerOr1* amplification were 5′-ATCTTCTTCGCATTCCACG-3′ and 5′-ATGAAAGTGATTGCCGCTC-3′, those for *AcerOr2* were 5′-GTGTTGTTCTGCTCCTGGCT-3′ and 5′-GGAAGGTGGTCGTGAAGTCG-3′, and those used to amplify *β-actin* were 5′-GTGACGACGAAGTAGCAGC-3′ and 5′-TGACCCATA CCGACCAT-3′. The reactions were run in a 7500 real-time PCR system (ABI, USA) by using SYBR® Select Master Mix. All reactions, including those for the *β-actin* control, were run in triplicate. Each reaction mixture (20 μL) consisted of 2 μL of template cDNA, 10 μL of SYBR® Premix Ex Taq II TM (2×), 0.4 μL of ROX Reference Dye II (50×), 0.8 μL of each of the forward and reverse primers (10 μM), and 6.0 μL of ddH_2O. The reaction conditions (two-step method) were as follows: pre-denaturation at 95 °C for 10 min, 1 cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 65 °C for 30–34 s. Melting curve analysis was conducted to assess primer specificity. The basal expression of *AcerOrs* was normalized to the expression of *β-actin* mRNA. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA levels of each target gene. All the RT reactions, including those for the *β-actin* controls, were performed in triplicate.

2.4. Western blot analysis

The cDNA sequences of *AcerOrs* were used to design and synthesize antigenic peptides [ENTTNYRNIHYKSD (14 aa) for AcerOr1 and NARYHQIAVK (10 aa) for AcerOr2]. These antigens were used for the generation of pAb_AcerOr1 and pAb_AcerOr2 antibodies by AbMax Biotechnology (Beijing, China), which were used for western blot analysis to confirm the expression of AcerOr1 and AcerOr2 in the sperm and testis of *A. cerana cerana*.

Total protein was quantified using the BCA Protein Assay Kit (Boster, Wuhan, China), according to manufacturer's instructions. The extracted proteins (40 μg per sample) were separated by 12% sodium dodecyl sulfate gel electrophoresis and then transferred onto a nitrocellulose filter membrane (Boster). The membranes were blocked for 1.5 h at 27 °C with 5% skimmed milk (Boster), and then incubated with rabbit polyclonal anti-AcerOr1, anti-AcerOr2 (1:1000 v/v); rabbit anti-CaM, rabbit anti-CaMKII, rabbit anti-p-CaMKII (1:1000 v/v; Bioworld Technology Co., Ltd., St. Louis Park, MN, USA), and mouse anti-β-actin (1:500 v/v) (Boster) antibodies overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit (1:5000 v/v; Boster) and goat anti-mouse (1:2000 v/v; Boster) IgG secondary antibodies for 2 h at 27 °C. Finally, bands were detected using the Super ECL Plus detection reagent (Boster) and were analyzed using Image Lab (Bio-Rad Laboratories, Hercules, CA, USA) and Image J (Version 1.49, National Institutes of Health, Bethesda, MD, USA).