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Ingestion of the epoxide hydrolase inhibitor AUDA modulates immune responses of the mosquito, *Culex quinquefasciatus* during blood feeding

Jiawen Xu, Christophe Morisseau, Jun Yang, Kin Sing Stephen Lee, Shizuo G. Kamita, Bruce D. Hammock*

Department of Entomology and UC Davis Comprehensive Cancer Center, University of California Davis, Davis, CA, USA

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ABSTRACT

Epoxide hydrolases (EHs) are enzymes that play roles in metabolizing xenobiotic epoxides from the environment, and in regulating lipid signaling molecules, such as juvenile hormones in insects and epoxy fatty acids in mammals. In this study we fed mosquitoes with an epoxide hydrolase inhibitor AUDA during artificial blood feeding, and we found the inhibitor increased the concentration of epoxy fatty acids in the midgut of female mosquitoes. We also observed ingestion of AUDA triggered early expression of defensin A, cecropin A and cecropin B2 at 6 h after blood feeding. The expression of cecropin B1 and gambicin were not changed more than two fold compared to controls. The changes in gene expression were transient possibly because more than 99% of the inhibitor was metabolized or excreted at 42 h after being ingested. The ingestion of AUDA also affected the growth of bacteria colonizing in the midgut, but did not affect mosquito longevity, fecundity and fertility in our laboratory conditions. When spiked into the blood, EpOMEs and DiHOMEs were as effective as the inhibitor AUDA in reducing the bacterial load in the midgut, while EETs rescued the effects of AUDA. Our data suggest that epoxy fatty acids from host blood are immune response regulators metabolized by epoxide hydrolases in the midgut of female mosquitoes, inhibition of which causes transient changes in immune responses, and affects growth of microbes in the midgut.

1. Introduction

Mosquitoes are vectors for a variety of devastating arthropod-borne pathogens, such as *Plasmodium*, dengue virus and West Nile virus. In order to understand the unique mosquito-host interactions and target new ways for disease control, recent studies have found that many blood-derived components are still biologically active when ingested by female mosquitoes. Blood components such as insulin, insulin-like factor, TGF-β can trigger the corresponding signal transduction pathways in mosquitoes and affect mosquitoes' capacity as disease vectors (Pakpour et al., 2013). Based on recent discoveries, more blood factors that are both functional in mammals and mosquitoes are likely to be reported.

Leukotoxins (EpOMEs) and epoxy eicosatrienoic acids (EETs) are epoxides of C-18 linoleic acid and C-20 arachidonic acid respectively, which are regular components in the mammalian blood. Elevated plasma level of EpOMEs is observed in human patients suffering from extensive burns (Hayakawa et al., 1990). EpOMEs are also associated with acute respiratory distress syndrome (ARDS) (Ozawa et al., 1988). Several studies suggest the diols of EpOMEs, the hydrolysis product of EpOMEs by the soluble epoxide hydrolase (sEH), are the active molecules responsible for the EpOME-associated toxicity (Moghaddam et al., 1997; Zheng et al., 2001). Epoxyeicosatrienoic acids (EETs) are another important class of epoxide substrates for the mammalian soluble epoxide hydrolase (Yu et al., 2000; Zeldin et al., 1993). EETs, as a group of potent chemicals called eicosanoids, have been extensively studied in terms of human health and drug development (Morisseau and Hammock, 2013). In mammalian systems, a relatively well-

Abbreviations: EH, epoxide hydrolase; JH, juvenile hormone; sEH, soluble epoxide hydrolase; JHEH, juvenile hormone epoxide hydrolase; EET, epoxyeicosatrienoic acid; EpOME, epoxy octadecenoic acid; DiHOME, dihydroxyoctadecanoic acid; DHET, dihydroxyeicosatrienoic acid; AUDA, 12-(3-adamantan-1-yl-ureido) dodecanoic acid.

* Corresponding author.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

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studied property of EETs is anti-inflammation, as well as vasodilation, angiogenesis, and analgesic effects (Morisseau and Hammock, 2013). 11,12-EET is reported to reduce tumor necrosis factor-α (TNF-α) induced inflammation by a mechanism that inhibits NF-κB activity (Node et al., 1999). 14,15-EET is also known to be anti-inflammatory (Morin et al., 2008). The role of EETs as anti-inflammatory chemical mediators is also supported by the use of EH inhibitors (Schmelzer et al., 2005; Smith et al., 2005). In mosquitoes, the Toll and Imd pathways are the major immune signaling pathways that are studied in the context of immunity and disease transmission. Both pathways are highly conserved and dependent on the NF-κB transcription factor to play crucial roles in anti-pathogen defense (Dong et al., 2011; Zou et al., 2011). Many immune genes are reported to be regulated by NF-κB, such as diptericin, cecropin, attacin, defensing and nitric oxide synthase (Dong et al., 2006; Hillyer and Estevez-Lao, 2010; Luna et al., 2006; Richman et al., 1997; Vizioli et al., 2000). However, it remains unknown whether EETs and other epoxy fatty acids are endogenous substrates for mosquito EHs, and whether they are chemical mediators that regulate immune responses in a conserved pathway as they do in mammals.

In mosquitoes, we recently reported mammalian sEH homologs, and epoxy fatty acids are endogenous molecules (Xu et al., 2014, 2015). We also found high EH activities on epoxy fatty acids in the midgut of female mosquitoes in comparison to other tissues, and proposed that the EH inhibitor AUDA could be used as a probe to investigate the function of EHs in the mosquito midgut (Xu et al., 2015).

In this study, we tested the hypothesis that epoxy fatty acids are immune response regulators derived from the host that are metabolized by the epoxide hydrolases in the mosquito midgut during blood feeding. We found that the ingestion of the EH inhibitor AUDA triggered early gene expressions of defensin A, cecropin A and cecropin B2 at 6 h post blood feeding. The ingestion also reduced the bacteria load in the midgut without causing any fitness loss in longevity, fertility and fecundity in our laboratory conditions. Our data supported our hypothesis, suggesting epoxy fatty acids are functional chemical mediators ingested by female mosquitoes, which play roles in regulating immune responses. Because the gut microbiota and the anti-bacterial responses affect the pathogen development in the mosquitoes (Cirimotich et al., 2011; Pan et al., 2012), the effects of EH inhibitor AUDA and related compounds on the biology of pathogens such as *Plasmodium* and Dengue virus should be tested in *Anopheles* and *Aedes* mosquitoes respectively.

2. Materials and methods

2.1. Mosquito rearing

The larvae of the mosquito *Culex quinquefasciatus* were reared in plastic water cups, and fed twice daily with a mixture of grounded fish food (TetraMin, Germany) and cat food (Purina, MO). Adults were fed 10% sucrose *ad libitum* soaked in cotton balls daily in mosquito cages (30 cm × 30 cm × 30 cm) in an insectary incubator at a temperature of 28 ± 1 °C and 80± 5% relative humidity.

2.2. Mosquito blood feeding

Before blood feeding, the 10% sucrose meal was removed from the rearing cages, and the mosquitoes were starved overnight. The next day, 5 ml of sheep blood (Quad Five, MT) was warmed to 37 °C in an incubator with gentle shaking. 5 μl of 10 mM AUDA in DMSO or 5 μl of DMSO was also added into the blood. The final concentration of the 'AUDA blood' is 10 μM AUDA, 0.1% DMSO (v/v). The control 'DMSO blood' contained only 0.1% DMSO (v/v). The sheep blood is routinely used to maintain the mosquito colony in the lab and contains serum and red blood cells. The concentrations of epoxy fatty acids in the serum were reported previously (Xu et al., 2015), and are comparable to the levels reported in other mammalian blood (Imig, 2012; Jiang et al., 2005, 2012). Female mosquitoes (4–7 days after eclosion) were allowed to feed for 30 min on sheep blood through a glass mosquito feeder, which was connected to a water circulator to keep the blood at a constant 37 °C.

2.3. Real-time quantitative PCR

The primers used in this study (Table S1) were designed by the Beacon Designer software (PREMIER Biosoft, CA) except for the bacterial 16S ribosomal RNA primers (Nadkarni et al., 2002). Total RNAs were extracted from 10 blood-fed female mosquitoes from each treatment using Trizol reagent (Invitrogen, MA) at various times post blood feeding. cDNA (from 1 μg total RNA) was synthesized by SuperScript® III reverse transcription (Life Technologies, NY). Real-time quantitative PCR was performed using SYBR® GreenER qPCR SuperMix Universal assay kit (Invitrogen, MA) on a 7500 Fast Real-time PCR System (Applied Biosystems, CA) under manufacturer's suggested conditions. Gene expression levels were normalized to the S7 ribosomal protein gene, and fold of change between the treatment groups was determined by the ΔΔCt method (Livak and Schmittgen, 2001).

2.4. Detection of the inhibitor AUDA or epoxy fatty acids in the midgut by LC-MS/MS

After mosquitoes were allowed to feed on artificial blood meal containing 10 μM AUDA, 0.1% DMSO (v/v) or 0.1% DMSO (v/v) only. Mosquito midguts were dissected at 6 h intervals and were immediately placed into 1.5 ml eppendorf tubes with 10 μl antioxidant solution (0.2 mg/ml of butylated hydroxytoluene and EDTA) and 10 μl of deuterated standards (Yang et al., 2009). 400 μl of methanol was added to each microfuge tube and the tubes were placed in a −80 °C freezer for 30 min. Subsequently, the midguts were homogenized with a plastic pestle and stored at a −80 °C freezer overnight. The next day the homogenates were centrifuged at 10,000*g* for 10 min and the supernatant was collected. The pellet was washed with 100 μl of ice-cold methanol, containing 0.1% of acetic acid and 0.1% of butylated hydroxytoluene. The samples were centrifuged again, and the supernatants were combined. The following sample preparation by solid phase extraction and analysis by LC-MS/MS was processed as previously described (Yang et al., 2009).

2.5. Longevity studies

Adult female mosquitoes were allowed to mate with males after emergence. After 4–7 days, the female mosquitoes were allowed to feed on an artificial blood containing 10 μM EH inhibitor AUDA, 0.1% DMSO or 0.1% DMSO only by a glass mosquito feeder at 37 °C for 30 min. Fully ingested females were transferred to a new cage, and were allowed to feed on 10% sucrose meals daily *ad libitum*. Daily mortality was recorded and dead mosquitoes were removed from the cage until all the mosquitoes died or censored. Analysis of survival curves was conducted by the Kaplan-Meier method (Kaplan E.L., 1958) and significant differences were determined by the nonparametric Wilcoxon test using the Prism 6 software (GraphPad, CA).