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Aldehyde Dehydrogenase 3 Converts Farnesal into Farnesoic Acid in the *Corpora Allata* of Mosquitoes

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Abstract

The juvenile hormones (JHs) play a central role in insect reproduction, development and behavior. Interrupting JH biosynthesis has long been considered a promising strategy for the development of target-specific insecticides. Using a combination of RNAi, *in vivo* and *in vitro* studies we characterized the last unknown biosynthetic enzyme of the JH pathway, a fatty aldehyde dehydrogenase (*AaALDH3*) that oxidizes farnesal into farnesoic acid (FA) in the *corpora allata* (CA) of mosquitoes. The *AaALDH3* is structurally and functionally a NAD⁺-dependent class 3 ALDH showing tissue and developmental-stage-specific splice variants. Members of the ALDH3 family play critical roles in the development of cancer and Sjögren-Larsson syndrome in humans, but have not been studied in groups other than mammals. Using a newly developed assay utilizing fluorescent tags, we demonstrated that *AaALDH3* activity, as well as the concentrations of farnesol, farnesal and FA were different in CA of sugar and blood-fed females. In CA of blood-fed females the low catalytic activity of *AaALDH3* limited the flux of precursors and caused a remarkable increase in the pool of farnesal with a decrease in FA and JH synthesis. The accumulation of the potentially toxic farnesal stimulated the activity of a reductase that converted farnesal back into farnesol, resulting in farnesol leaking out of the CA. Our studies indicated *AaALDH3* plays a key role in the regulation of JH synthesis in blood-fed females and mosquitoes seem to have developed a “trade-off” system to balance the key role of farnesal as a JH precursor with its potential toxicity.

Keywords

Aedes aegypti; mosquito; juvenile hormone; *corpora allata*; aldehyde dehydrogenase 3; RNAi; aldo keto reductase; farnesal

1. Introduction

Juvenile hormone (JH) is a key regulator in insect morphogenesis and reproduction (Goodman and Cusson, 2012). For this reason, interfering with its biosynthesis has long been considered a promising strategy for the development of target-specific insecticides (Cusson et al., 2013). The biosynthetic pathway of JH III in the *corpora allata* (CA) of the Dengue mosquito *Aedes aegypti* involves 13 discrete enzymatic steps (Nouzova et al.,

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2011); the early steps follow the mevalonate pathway to form farnesyl pyrophosphate (FPP) (Belles et al., 2005). During the late steps, FPP is transformed sequentially to farnesol, farnesal, farnesoic acid (FA), methyl farnesoate and JH III (Goodman and Cusson, 2012). The last 2 enzymes of the pathways have been identified and used to screen for inhibitors (Shinoda and Itoyama, 2003; Helvig et al., 2004); however, the oxidation of farnesal to FA remains as one of the less understood steps in JH synthesis, and it has been predicted to be catalyzed by an aldehyde dehydrogenase (Baker et al., 1983; Goodman and Cusson, 2012). The aldehyde dehydrogenase superfamily (ALDH, E.C. 1.2.1.X) comprises a cluster of evolutionary related sequences with a wide phylogenetic distribution (Dayhoff, 1976). It includes NAD(P)⁺ dependent enzymes catalyzing the oxidation of a broad spectrum of aldehyde substrates to their corresponding acids (Pietruszko, 1983). The human ALDH3A2 is able to convert long-chain aldehydes, including farnesal, to their related acids (Lloyd et al., 2007); mutations of ALDH3A2 gene causes Sjögren-Larsson syndrome, a disorder associated with impaired fatty alcohol oxidation due to deficient activity of fatty alcohol oxidoreductase and its consequent oxidation to fatty aldehyde and fatty acid (Rizzo and Craft, 1991; De Laurenzi et al., 1996; Kelson et al., 1997). Farnesal oxidation in the CA was first revealed in *Drosophila melanogaster* using an *in vitro* colorimetric assay (Madhavan et al., 1973). Subsequently the conversion of 2*E*- and 2*Z*- farnesal to FA by CA homogenates of the adult female sphinx moth, *Manduca sexta* was reported (Baker et al., 1983). Further characterization of the CA ALDH has been hindered by the minute size of the endocrine gland. Recently, we described a new and sensitive assay for the JHs and their precursors using fluorescent tags (Rivera-Perez et al., 2012); this assay allowed the quantification of farnesal and FA pools in the CA, as well as ALDH enzymatic activities from CA extracts. Here, we report the molecular and biochemical characterization of an *A. aegypti* ALDH3 (*AaALDH3*) expressed in the CA. Our results suggest that in the female of the Dengue mosquito *A. aegypti*, this enzyme plays a critical role in the regulation of JH biosynthesis after blood-feeding; in CA of blood-fed females the low catalytic activity of *AaALDH3* limited the flux of precursors and caused a major increase in the pool of farnesal with a decrease in FA and JH synthesis. The accumulation of the potentially toxic farnesal stimulated the activity of a reductase that converted farnesal back into farnesol, resulting in farnesol leaking out of the CA. This aldehyde scavenging enzyme prevents aldehyde toxicity in CA cells and plays a key role in farnesal homeostasis.

2. Materials and methods

2.1. Insects

A. aegypti of the Rockefeller strain were reared as described by Nouzova et al. (2011).

2.2. Identification of two farnesal dehydrogenase genes in *Aedes aegypti*

We screened the *A. aegypti* genome (Assembly AaegL1) for homologue sequences to the Human ALDH3A2 (P51648) that has been reported to transform farnesal into FA (Lloyd et al., 2007). The search revealed an area in the supercontig 1.662 containing three predicted genes in close proximity (AAEL012161, AAEL012162 and AAEL012165), which showed more than 50% protein identity with the human ALDH3A2 gene. Closer examination of this contig disclosed that there were only two genes, with AAEL012161 and AAEL012165 being parts of the same gene. There was also one predicted gene in the supercontig 1.417 with a DNA sequence almost identical to AAEL012162 (with a few point mutations). Primers were designed to amplify the full coding sequences of the two genes from cDNA of *corpora allata* (Supplemental Table 1). They were named *AaALDH3-1* and *AaALDH3-2*. Sequencing of the cDNAs revealed five different transcripts from the two different genes. The gene *AaALDH3-1* produces four different transcripts (A, B, C, and D) and the gene

AaALDH3-2 produces only one transcript. The individual transcripts from gene *AaALDH3-1* differ only in exon six (Supplemental Fig. 1).

2.3. Secondary structure and phylogenetic analysis

Secondary structures for ALDHs were predicted using PSIPRED version 2.5 (Jones, 1999) and transmembrane helices using TMHMM server version 2.0 (Nugent and Jones, 2009). A maximum-likelihood tree was built using MEGA software version 5.1 (Tamura et al., 2011), with a bootstrapping value of 1000. A pairwise deletion method was selected for the gap/missing data.

2.4. Expression of recombinant AaALDH3s

AaALDH3 cDNAs were expressed in *E. coli* cells as described by Mayoral et al. (2009b). Recombinant His-tagged proteins were identified by Western blot using a mouse anti-His antibody as described by Mayoral et al. (2009b).

2.5. Enzyme assays

Bacterial crude extract containing overexpressed proteins were used to test substrate specificity and cofactor requirements. The ALDH activity assay was optimized based on a protocol described by Lloyd et al (2007). Reaction mixtures (750 μ L) contained 50 mM glycine buffer (pH 9.5), 2 mM NAD⁺ and 50 μ M substrate (octanal, decanal or farnesal). Reactions were started by the addition of 5 μ g of bacterial extracts. After 2 h incubation at 37 °C, reactions were stopped with 500 μ L of hexane, vortexed 1 min and centrifuged for 10 min at 14,000 *g* and 4 °C; supernatants were used for analysis of production of octanoic acid, decanoic acid and FA by reversed-phase high performance liquid chromatography (RP-HPLC). Negative controls included boiled enzyme, no enzyme or bacterial cells without constructs. ALDH and aldo-keto reductase (AKR) activities in mosquito CA were measured by HPLC coupled to a fluorescent detector (HPLC-FD), monitoring the production of FA and farnesol respectively. The AKR activity assay was optimized based on a protocol described by Endo et al (2009). Glands were dissected in buffer solution (50 mM glycine buffer pH 9.5 for ALDH and 0.1 M phosphate buffer pH 7.4 for AKR). CA were homogenized for 1 min, sonicated 3 min and centrifuged at 4 °C and 10,000 *g* for 10 min. Supernatant were recovered and used as crude extracts for ALDH activity assays as described before; for AKR assays, the reaction mixture consisted of 0.1 M phosphate buffer, 0.1 mM NADPH and 50 μ M farnesal, both reactions mixtures were incubated 1 h at 37 °C. The reactions products were recovered as described before and labeled with AABD-SH (farnesoic acid) and DBD-COCl (farnesol) for HPLC-FD analysis (Rivera-Perez et al., 2012).

2.6. Precursor pool quantification

Farnesal, farnesol and FA quantification by HPLC-FD and mass spectrometry characterizations were done as described by Rivera-Perez et al. (2012).

2.7. RNAi experiment

Specific target sequences for dsRNA synthesis were designed for each splice variant of *AaALDH3-1* by selecting regions in the splice variant-specific exons. We also designed dsRNA for *AaALDH3-2* as well as dsRNA against a common region to all splice variants of *AaALDH3-1* (*AaALDH3-1-RA*, *AaALDH3-1-RB*, *AaALDH3-1-RC*, *AaALDH3-1-RD*) and a Yellow Fluorescent Protein (YFP), that was used as control. Targeted regions were amplified using the primers included in Supplemental Table 1. dsRNA was synthesized using a Megascript RNAi kit (Ambion), and 1.6 μ g of dsRNA were injected intrathoracically to 0 h female mosquitoes using a Drummond Nanoject II microinjector and

a micromanipulator. The effect of dsRNA was evaluated 4 days after injection, a time selected based on the analysis of dsRNA depletion experiments.

2.8. Quantitative real-time PCR (qPCR)

RNA isolation and PCR were performed as described by Nouzova et al. (2011). The primers and probes for the house keeping gene 60S ribosomal protein rpl32 and *AaALDH3* transcripts are included in Supplementary Table 1.

2.9. Statistical analysis

Data were analyzed for statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA). The results were expressed as means $\pm$ S.E.M. Significant differences ($P < 0.05$) were determined with a one tailed students t test performed in a pair wise manner or one way ANOVA followed by a comparison of means (Turkey's test).

3. Results

3.1. Identification of two *A. aegypti* ALDH3s

Using the sequence of a Human ALDH3 that converts farnesal into FA (ALDH3A2) (De Laurenzi et al., 1996) we screened the *A. aegypti* genome (Vectorbase) (Lawson et al., 2009). Two genes showing over 50% amino acid sequence identity were revealed: *AaALDH3-1* that produces four different transcripts differing only in exon six (A, B, C, and D), and *AaALDH3-2* with only one transcript (Supplemental Fig. 1 and Supplemental Table 2). Amino acid sequence alignments of *A. aegypti*, *D. melanogaster* and human ALDH3s revealed conserved residues involved in chain folding, formation of the active site funnel, substrate binding, cofactor binding, stabilization of the transition state during catalysis and aldehyde oxidation (Liu et al., 1997; Hempel and Wang, 1997; Lloyd et al., 2007; Holmes and Hempel, 2011) (Supplemental Fig. 2). A phylogram was generated using ALDH3 orthologues found in insects and vertebrates (Supplemental Table 2 and Supplemental Fig. 3). In most insect we found a single orthologue of *AaALDH3*; Culicidae species and *Tribolium* being the exceptions. One transcript was found in primitive insects and ticks, with holometabola exhibiting at least two alternative transcripts.

3.2. All *AaALDH3* variants converted farnesal into FA

Five recombinant His-tagged *AaALDH3*s, representing *AaALDH3-2* and the 4 splice variants of *AaALDH3-1* (PA, PB, PC and PD) were overexpressed in *E. coli*. Recombinant proteins were identified from bacteria extracts using an anti-His antibody. *AaALDH3*s catalytic activities on aldehyde substrates were measured by RP-HPLC. *AaALDH3*s oxidized farnesal to FA in the presence of NAD⁺, and NADP⁺ could not substitute for it (Table 1). *AaALDH3-1-PC* had the highest activity with farnesal. All of the enzymes oxidized octanal and decanal with the exception of *AaALDH3-1-PA* which did not have activity with decanal. Reaction products were analyzed by LC-MS. Octanoic, decanoic and farnesoic acids were identified based on the diagnostic ions (m/z 143.2, 171.2, 235.3 respectively).

3.3. The CA has NAD⁺-dependent farnesal dehydrogenase activity

ALDH activities were detected in CA extracts from adult female mosquitoes having 3 distinct JH biosynthetic rates: basal activity (0 h or newly emerged adult), high activity (24 h sugar-fed) and suppressed activity (24 h after blood feeding) (Fig. 1A). In the presence of an excess of farnesal and NAD⁺, highly active glands produced 10 times more FA than suppressed glands. Remarkably, the CA with basal activity from newly emerged females, that produced only 12 fmol/h of JH, had almost 20 times more ALDH activity than the

highly active glands that produced 43 fmol/h of JH. NAD^+ was also absolutely required for the activity of the CA extracts and NADP^+ did not substitute for it (Fig. 1B). Extracts of CA with high and suppressed JH biosynthetic activity were incubated with an excess of NAD^+ in the presence or absence of exogenous farnesal. Production of FA was significantly reduced when CAs were not stimulated with exogenous farnesal (Fig. 1C).

3.4. Tissue- and developmental-stage-specific expression of AaALDH3s

Transcript abundance for the *AaALDH3* genes, including the splice variants (RA, RB, RC and RD, where R stands for RNA), was quantified by qPCR. The distribution of transcript was unique for each tissue, with each having one dominant type of transcript: *AaALDH3-1-RA* in ovaries, *AaALDH3-1-RB* in Malpighian tubes, *AaALDH3-1-RC* in hindgut and *AaALDH3-1-RD* in nervous tissue. The *AaALDH3-2* gene had comparatively lower levels of transcript (Fig. 2A). CA at all sampling times presented the five transcripts, with their relative proportions changing during development; the major change happening between 0 and 24 h after adult female emergence, where *AaALDH3-1-RA*, which prevails in pupa, was replaced by *AaALDH3-1-RC* (Fig. 2B). None of the transcript profiles followed closely the changes of JH synthesis (Li et al., 2003); *AaALDH3-1-RC* was the closest as its transcript levels increased 2.6-fold 24 h after mosquito emergence and then gradually decreased with age in sugar-fed females. Transcript levels in CA for all the *AaALDH3* variants modestly increased after blood feeding.

3.5. In vivo depletion of AaALDH3 mRNAs reduced the FA pool in the CA

Injection of dsRNAs resulted in a significant reduction of *AaALDH3-1* and *AaALDH3-2* mRNAs (~ 80%) (Supplemental Fig. 4). Treatment with dsRNA for the *AaALDH3-1* gene resulted in stronger reductions of FA and JH than treatment with dsRNA for the *AaALDH3-2* gene or YFP; among the 4 isoforms, *AaALDH-1-PC* had the highest reduction of the FA pool (75%), but had a less pronounced effect on JH synthesis (Fig. 3). *In vivo* depletion of all isoforms of *AaALDH3-1* had a strong effect on the ALDH activity of CA extracts; extracts of *AaALDH3-1* silenced mosquitoes had only 1% of activity of YFP treated animals, suggesting that the activity of *AaALDH3-1* is mainly responsible for the conversion of farnesal to FA in the CA of mosquitoes (Fig. 3).

3.6. Reduction of AaALDH3 activity results in accumulation of farnesal and conversion back to farnesol that leaks outside the CA

The endogenous pools of farnesol, farnesal and FA in CA dissected from newly emerged adult females (basal activity), 24 h sugar-fed females (24 h SF) (high activity) and females 24 h after blood feeding (24 h BF) (suppressed activity) were markedly different (Fig. 4). Pools in newly emerged adult females were larger than those of sugar or blood-fed females; interestingly, the CA of blood-fed females that had reduced ALDH3 activity showed a large increase in the size of the farnesal pool, suggesting a potential bottleneck in the flux of precursors between farnesal and FA; surprisingly these glands had a very small farnesol pool. We hypothesized that the massive accumulation of the potentially toxic farnesal could stimulate the activity of a farnesal reductase or another enzyme that could convert farnesal back into farnesol, leading to leaking of farnesol outside the CA. This was tested by incubating CAs dissected from sugar and blood-fed females in the presence of exogenous farnesal, and evaluating the amount of farnesol that leaked in the incubation medium. The results confirmed our hypothesis; a significant amount of farnesol was detected leaking from un-stimulated or farnesal-stimulated CAs from blood-fed females, while only supra-stimulated (200 μM) CA from sugar-fed females showed a significant leakage of farnesol (Fig. 5A). The human aldo-keto reductase (AKR1B10), which can process farnesal to farnesol had two close orthologues in *A. aegypti*. These two genes (*AaAKR-1* and *-2*) are expressed in the CA (Supplemental Fig. 5). We measured farnesal NADPH-dependent

reductase activity in the CA of sugar-fed and blood-fed females; reductase activity was significantly increased in the CA of 24 h after blood-feeding (Fig. 5B).

4. Discussion

4.1. AaALDH3s are long chain aldehyde dehydrogenases that oxidize farnesal to farnesoic acid in the CA of mosquitoes

We have identified the last unknown enzyme of the JH biosynthetic pathway in insects. *AaALDH3* is structurally and functionally a NAD⁺-dependent ALDH3. Fatty aldehyde dehydrogenases are NAD(P)⁺-dependent oxidoreductases involved in the metabolism of fatty aldehydes (Kelson et al., 1997; Lloyd et al., 2007). They play key roles in the generation of fatty alcohols and fatty acids; as well as in the elimination of toxic biogenic and xenobiotic aldehydes, such as those produced by oxidative damage of glycerolipids or during protein deprenylation (Jakoby and Ziegler, 1990; Rizzo and Craft, 1991; Tschantz et al., 2001). Mammalian ALDHs are classified according to their subcellular distribution, kinetic properties and primary sequences. Class 1 ALDHs are cytosolic and class 2 are mitochondrial, both classes acting on short-chain aliphatic aldehydes (Lindahl, 1992). Class 3 includes cytosolic, mitochondrial and microsomal enzymes, with some microsomal ALDH (ALDH10 or FALDH) processing long chain-aldehydes (E.C. 1.2.1.48) (Kelson et al., 1997). All ALDHs contain a classical Rossmann-fold for nucleotide binding, a catalytic domain and a bridging domain (Liu et al., 1997). Class 3 ALDHs act as dimers and are characterized by a hydrophobic sequence of 17 residues at the C-terminus, which works as a membrane anchor (Liu et al., 1997; Miyauchi et al., 1991). During the biosynthesis of JH in the CA of mosquitoes, farnesol is oxidized to farnesal by a NADP⁺-dependent short-chain dehydrogenase (*AaSDR-1*) (Mayoral et al., 2009a), while farnesal is converted into FA by a NAD⁺-dependent aldehyde dehydrogenase. In contrast to the ALDH3 described in other organisms (Liu et al., 1997; Rizzo et al., 2001; Ashibe et al., 2007), the *AaALDH3* isozymes are all microsomal and contain an ER targeting sequence on the carboxyl-terminus (Miyauchi et al., 1991; Masaki et al., 1994); in the CA this posttranslational signaling might play an important role in the subcellular localization of the catalytic reaction and the transfer of FA to the next enzyme of the pathway, methyl transferase (JHAMT) (Mayoral et al., 2009b). All recombinant *AaALDH3* isozymes were able to oxidize short and long-chain aldehydes, including farnesal. In vertebrates, ALDH3s have activity against a broad range of aliphatic aldehydes ranging from 2 to 24 carbons (Mitchell and Petersen, 1987; Kelson et al., 1997), however, only the human ALDH3A2 has been shown to oxidize farnesal (Lloyd et al., 2007). Class 3 ALDHs are the only family with the ability to use either NAD⁺ or NADP⁺; however, *in vivo* they might only employ NAD⁺ as cofactor (Perozich et al., 2000). As described for human ALDH3, the recombinant *AaALDH3* isozymes had an absolute requirement for NAD⁺, and NADP⁺ was unable to substitute for it (Kelson et al., 1997; Lloyd et al., 2007). Mosquito CA homogenates also required NAD⁺, in agreement with studies using CA extracts from *M. sexta*, that indicated that both 2*E*- and 2*Z*- farnesal isomers could be oxidized using NAD⁺ as cofactor (Baker et al., 1983). *AaALDH3* displayed tissue-specific and developmentally-specific alternatively spliced variants. In most insects we found a single orthologue of *AaALDH3*; Culicidae species and *Tribolium* being the exceptions. One transcript was found in primitive insects and ticks, with holometabola exhibiting at least two alternative transcripts. The presence of more than one isozyme with several isoforms capable of catalyzing the oxidation of farnesal in mosquitoes might have facilitated the evolution of more efficient substrate specificities, as well as a better tissue and developmental regulation. The tissue distribution was unique for each transcript and each tissue had one dominant type of transcript suggesting that each isozyme has specific functions and production of each transcript is highly regulated at the tissue level. Analysis of isoform-specific (IS) developmental changes in transcripts levels, IS-RNAi depletion experiments and IS-enzymatic activities in CA suggest that: 1) all the isoforms might play a

role in the conversion of farnesal into FA, 2) *AaALDH-1-PA* might be the most active variant in late pupae, responsible for the large pool of FA existent in the CA of newly emerged females, 3) *AaALDH-1-PC* seems to be the dominant form in adult female CA; it metabolizes farnesal into FA more effectively, changes in transcript levels correlated better with changes in JH synthesis (Li et al., 2003) and specific depletion of its transcripts had the strongest effect on farnesal oxidation. Even if the silencing of *AaALDH3* was effective and conversion of FAL into FA was limited, the moderate effect of the RNAi experiments on JH synthesis *in vivo* might be related to the existence of a relative large pool of FA present in those glands.

4.2. Oxidation of farnesal is a rate limiting step in JH synthesis after blood feeding

JH is a key regulator of ovarian maturation in mosquitoes (Klowden, 1997). Newly emerged females have a CA with low biosynthetic activity, but they produce high amounts of JH a day later; blood feeding results in a striking decrease in JH synthesis (Li et al., 2003). The rate of JH synthesis is regulated by the rate of flux of isoprenoids in the pathway that depends on a complex interaction of changes in precursor pools, enzyme levels and external regulators (Schooley and Baker, 1985). Changes in the nutritional status in female mosquitoes (Caroci et al., 2004; Noriega, 2004) as well as the manipulation of individual precursor pool concentrations (e.g. farnesol, farnesal and FA) affect the rates of JH synthesis (Nouzova et al., 2011). Until now, simple methods for detailed analysis of enzymatic activities derived from insect CA were not readily accessible; therefore the activities of the biosynthetic enzymes have been mainly studied using recombinant proteins (Shinoda and Itoyama, 2003; Helvig et al., 2004; Mayoral et al., 2009a and 2009b). Using fluorescence approaches, we were able to measure the changes in the precursor pools sizes and the production of FA by aldehyde dehydrogenases from CA extracts. Having detected remarkable differences among basal, highly active and depressed glands suggests that the molecular bases for JH regulation are quite unique at different times during the reproductive cycle of an adult female mosquito. A nutritional-dependent post-emergence JH increase initiates previtellogenic ovarian development; a newly emerged female mosquito needs first to assess the size of teneral nutritional reserves before it stimulates an increase in the synthesis of JH from basal to high levels (Caroci et al., 2004; Noriega, 2004; Hernandez-Martinez et al., 2007). The CA of the newly emerged female has a very active farnesal dehydrogenase and large pools of farnesal and FA, however produces only basal levels of JH, suggesting that factors from the brain might be needed to increase the activity of the last two enzymes of the pathway (JHAMT and/or epoxidase), or there exists an alternative regulatory mechanism that prevents FA to be readily converted into MF and/or JH. On the other hand, when pools of farnesal and FA and *AaALDH3* enzymatic activities were compared in highly active (24h SF) and suppressed glands (24h BF), significant differences of *AaALDH3* activities strongly suggested that the enzyme concentration and not the farnesal pool was a limiting factor, indicating *AaALDH3* is a key regulator of JH biosynthesis after a blood meal. In suppressed CA (24h BF) the low catalytic activity of *AaALDH3* limited the flux of precursors and caused a remarkable increase in the farnesal pool. Human cells deficient of ALDH3A2 accumulate fatty aldehydes; in fibroblasts fatty aldehydes are reduced back to fatty alcohols (Rizzo and Craft, 2000). Aldehydes are highly reactive and toxic for cells (Voziyan et al., 1995; Rizzo et al., 2001), however long-chain alcohols, such as farnesol, have a high partition coefficient for biological membranes and intercalate into cellular membranes rather than existing free in solution (Franks and Lieb., 1986). The excess of farnesal in the CA should be a good substrate for aldehyde reductases that could reduce farnesal to farnesol in presence of NADPH (Endo et al., 2009). The first evidence for this type of CA homeostatic mechanism came from the detection of large amounts of farnesol in the medium when glands were incubated with high farnesal concentrations, suggesting that farnesol was leaking out of the glands. This reaction might

be catalyzed by an orthologue of the human aldo-keto reductase (AKR1B10), which has been proven can process farnesal to farnesol (Endo et al., 2009). Under conditions when farnesal accumulated inside the CA, we detected an increase in the activity of CA reductase, as well as a rise in the amount of farnesol leaking from the gland. Based on those results, we have developed a model describing the role of *AaALDH3* in the flux regulation in suppressed and highly active mosquito glands (Fig. 6).

Separation of individual enzymatic reaction and precursor pools by cellular membranes could impose constraints on the isoprenoid flux by disrupting pathway connections that otherwise would be possible based on simple reaction mechanisms. Metabolic enzymes that catalyze a series of successive reactions can form complexes on membranes or cytoskeletal structures (Newman and Chappell, 1999). Such metabolic enzyme complexes are called 'metabolons', functioning as metabolic channels that facilitate metabolite flux to committed end products (Moller, 2010). Metabolons can 'move' metabolites more efficiently through the pathway and limit availability of potential common metabolite intermediates to other branches of the network (Jorgensen et al., 2005). Metabolon formation normally involves specific interactions between several 'soluble' enzymes that might be anchored to a membrane either by membrane-bound structural proteins that serve as 'nucleation' sites for metabolon formation or by membrane-bound proteins; *AaADLH3* or epoxidase could serve that role in the CA of mosquitoes. In vertebrates farnesal dehydrogenase closely interacts with farnesol dehydrogenase, forming a complex called "alcohol:NAD⁺ oxidoreductase" (FAO); responsible for the sequential oxidation of fatty alcohol to fatty acids (Ichihara et al., 1986; Rizzo et al., 1991). A similar complex including the two oxidoreductases, the JHAMT and epoxidase might be present in the CA of mosquitoes, channeling precursors efficiently in the JH pathway. *In vitro* experiments have shown that several intermediates in the pathway (e.g. mevalonate, farnesol, farnesal, FA) are incorporated into the CA and stimulate JH synthesis (Tobe and Stay, 1985; Piulachs and Couillaud, 1992; Nouzova et al., 2011). It is puzzling that the CA of a newly emerged mosquito female that has a very large FA pool but limited JH synthesis is strongly stimulated by exogenous supply of FA (Nouzova et al., 2011). These results suggest differences in the channeling of "endogenous" and "exogenous" FA derived pools. Similarly, while silencing of *AaADLH3* variants resulted in dramatic decreases of the farnesal dehydrogenase activity (sometimes as much as 99%), the FA pool was still large enough to supply sufficient substrate to the JHAMT to synthesize 15 fmol/JH/h. The use of fluorescent approaches to quantify endogenous pools, enzymatic activities and the effect of pool perturbations in CA of mosquitoes is a promising approach to unravel the regulatory points in flux of precursors in the JH pathway. Our results suggest that under specific physiological conditions *AaSDR-1* and *AaALDH3* are key regulatory enzymes in the JH pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Aldehyde dehydrogenase 3 (ALDH3) converts farnesal (FAL) into farnesoic acid (FA) in the mosquito corpora allata (CA).
- The knockdown of the aldehyde dehydrogenase gene abolished ALDH3 activity and impaired JH synthesis.
- In the CA of blood-fed females, the low activity of ALDH3 limited the flux of precursors and impaired JH synthesis.
- Accumulation of farnesal in the CA stimulated the conversion of farnesal to farnesol by an aldehyde keto reductase (AKR1).
- Aldehyde dehydrogenase 3 plays a key role in the regulation of juvenile hormone (JH) synthesis in blood-fed females.

Fig. 1.

Aldehyde dehydrogenase activity in CA extracts. A) Farnesal dehydrogenase activity: Extracts of CA dissected from newly emerged females (0 h), 24 h after emergence (24h SF) and 24 h after blood feeding (24h BF) were incubated for 1 h in the presence of an excess of farnesal and NAD⁺. B) Cofactor requirements: Extracts of CA dissected from 24 SF were incubated for 1 h in the presence of an excess of farnesal and NAD⁺ or NADP⁺. C) Cofactor requirements: Enzymatic activities from extracts of CA dissected from sugar-fed (24h SF) and blood-fed (24h BF) mosquitoes were measured in the presence of an excess of exogenous NAD⁺ with or without supply of an excess of exogenous farnesal. Bars represent the means $\pm$ S.E.M. of two replicates of extracts from groups of 10 CA. Asterisks denote significant difference (unpaired t-test, ***P< 0.001, ** P< 0.01). Different letters above the columns indicate significant differences among treatments (one way ANOVA p < 0.05, with Tukey test of multiple comparisons).

Fig. 2.

Tissue specific and developmental expression of *AaALDH3*s: A) Tissue specific expression of *AaALDH3* mRNA: All tissues were dissected from 3-day-old sugar-fed females, except for testis and accessory glands dissected from 3-day-old sugar-fed males. BR: brain; SG: salivary gland; TG: thoracic ganglia; VG: ventral ganglia; HT: heart; FB: fat body; MG: midgut; HG: hindgut; MT: Malpighian tubules; OV: ovaries; AG: accessory gland and CA: corpora allata. Each data point is the average of two independent biological replicates of 10–20 tissue samples evaluated by triplicate. B) Developmental expression of *AaALDH3*: mRNA was isolated from CA of pupae 24 h (–24) and 6 h (–6) before adult eclosion, newly emerged adult female (0h), sugar-fed females 24, 48, 72 and 96h after eclosion (24hS, 48hS, 72hS and 96hS) and females 24, 48 and 72h after blood feeding (24hB, 48hB and 72hB). *AaALDH3* variants are: *AaALDH3*-1-RA: purple, *AaALDH3*-1-RB: orange, *AaALDH3*-1-RC: red, *AaALDH3*-1-RD: green and *AaALDH3*-2: blue. Each data point is the average of two independent biological replicates of 20 CA evaluated by triplicate. *AaALDH3* mRNAs are expressed as copy number of mRNA/10,000 copies of rpl32 mRNA. For clarity sake the error bars were omitted. Variances among triplicates were not significant.

Fig. 3.

dsRNA mediated knockdown of AaALDH3. Newly emerged female mosquitoes were injected with AaALDH3 or YFP dsRNA; 4 days later FA and JH levels were evaluated. A) FA pool sizes in CA: FA levels are expressed as % of the YFP controls. 1: all variants of AaALDH3-1, 1-PA, 1-PB, 1-PC and 1-PD: specific variants of AaALDH3-1; 2: AaALDH3-2 (P is for protein). Bars represent the means $\pm$ S.E.M. of two replicates of 10 CA. B) FA pool sizes in CA: levels are expressed as fmol/CA. Bars represent the means $\pm$ S.E.M. of two replicates of 10 CA. C) JH synthesized in vitro: CA were dissected from females injected with AaALDH3 (1 or 1-PC) or YFP dsRNA and incubated in vitro for 4 h. JH was evaluated by HPLC-FD. Bars represent the means $\pm$ S.E.M. of four replicates of 4 CA. D) Farnesal dehydrogenase activity: enzymatic activity by extracts of CA dissected from mosquitoes injected with AaALDH3 or YFP dsRNA. Bars represent the means $\pm$ S.E.M. of two replicates of 10 CA. Values are expressed as fmol of FA produced by CA/h. Different letters above the columns indicate significant differences among treatments (A, B and C: one way ANOVA $p < 0.05$, with Tukey test of multiple comparisons; D: unpaired t-test $P < 0.05$).

Fig. 4.

Changes of precursor pools and JH synthesis in *A. aegypti* CA. Relationship between JH synthesis and the sizes of farnesol, farnesal and FA pools in CA of newly emerged female mosquitoes (0 h), 24 h sugar-fed (24 h SF) and 24 h after blood feeding (24 h BF). Left Y axis: farnesol, farnesal and FA pools expressed as fmol/CA. Right Y axis: JH biosynthesis in vitro expressed as fmol/CA/h. Bars represent the means $\pm$ S.E.M. of two independent replicates of groups of 10 CA

Fig. 5.

Reverse synthesis of farnesol and leaking out of the CA: A) Stimulation of farnesol synthesis by exogenous addition of farnesol: The amount of farnesol leaking was evaluated in the medium from CA incubated for 4 h in vitro without (control) or with the addition of 40 and 200 μ M of farnesol. Farnesol leaking from CA dissected from 24 h sugar-fed (filled bar) and 24 h blood-fed (empty bar) females are expressed in fmol/CA. Bars represent the means $\pm$ S.E.M. of two independent biological replicates of 4 CA. Unpaired t-test; $P < 0.001$. B) Aldo-keto reductase activity in CA extracts: Extracts of CA dissected from 24 h sugar-fed (24h SF) and 24 h blood-fed (24h BF) females were incubated for 1 h in the presence of an excess of farnesol and NADPH. Bars represent the means $\pm$ S.E.M. of two replicates of groups of 10 CA. (unpaired t-test, *** $P < 0.001$).

Fig. 6. Model for the role of *AaALDH3* in the regulation of JH synthesis. (A) Highly active CA from 24 h sugar-fed female. (B) CA with suppressed activity from 24 h blood-fed female. FOL: farnesol; FAL: farnesal; FA: farnesoic acid; JH: juvenile hormone; SDR: short chain dehydrogenase; ALDH3: aldehyde dehydrogenase; AKR1: aldo-keto reductase; NADP^{aldehyde}: nicotinamide-adenine diphosphate (oxidized form); NAD⁺: nicotinamide-adenine dinucleotide (oxidized form); NADH: nicotinamide-adenine dinucleotide (reduced form). Size of circles and arrows denote the sizes of precursor pools and the rates of fluxes. The large circle represents the CA.

Table 1

Specific activity of recombinant *Aa*ALDH3s.

Substrate	$\mu\text{mol mg protein}^{-1} \text{ min}^{-1}$					
	<i>Aa</i> ALDH3-1-PA	<i>Aa</i> ALDH3-1-PB	<i>Aa</i> ALDH3-1-PC	<i>Aa</i> ALDH3-1-PD	<i>Aa</i> ALDH3-2	
Octanal ($\text{C}_8:0$)	152.69 $\pm$ 72.3	116.84 $\pm$ 45.7	214.62 $\pm$ 45.24	168.07 $\pm$ 52.3	203.86 $\pm$ 6.08	
Decanal ($\text{C}_{10:0}$)	0	287.07 $\pm$ 73.0	362.35 $\pm$ 66.00	103.70 $\pm$ 72.0	132.51 $\pm$ 6.24	
Farnesal ($\text{C}_{15:0}$)	272.93 $\pm$ 12.20	269.14 $\pm$ 10.8	468.24 $\pm$ 52.44	268.01 $\pm$ 11.6	209.22 $\pm$ 1.07	
NADP ⁺ ^a	0	7.08 $\pm$ 0.05	7.13 $\pm$ 0.04	0	0	

^aNADP⁺ activity was determined with farnesal as substrate.