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Publication Date

2018-05-01

DOI

10.1016/j.ibmb.2018.02.003

Peer reviewed

HHS Public Access

Author manuscript

Insect Biochem Mol Biol. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Insect Biochem Mol Biol. 2018 May ; 96: 19–26. doi:10.1016/j.ibmb.2018.02.003.

HR38, an ortholog of NR4A family nuclear receptors, mediates 20-hydroxyecdysone regulation of carbohydrate metabolism during mosquito reproduction

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Abstract

The *Aedes aegypti* mosquito is the principal vector for many dangerous human viral diseases. Carbohydrate metabolism (CM) is essential for supplying the energy necessary for host seeking, blood digestion and rapid egg development of this vector insect. The steroid hormone 20-hydroxyecdysone (20E) and the ecdysone receptor (EcR) are important regulators of CM, coordinating it with female reproductive events. We report here that the NR4A nuclear receptor AHR38 plays a critical role in mediating these actions of 20E and EcR. *AHR38* RNA interference (RNAi) depletion in female mosquitoes blocked the transcriptional activation of CM genes encoding phosphoglucomutase (PGM) and trehalose-6-phophate synthase (TPS); it caused an increase of glycogen accumulation and a decrease of the circulating sugar trehalose. This treatment also resulted in a dramatic reduction in fecundity. Considering that these phenotypes resulting from *AHR38* RNAi depletion are similar to those of *EcR* RNAi, we investigated a possible connection between these transcription factors in CM regulation. *EcR* RNAi inhibits the *AHR38* gene expression. Moreover, the 20E-induced EcR complex directly activates *AHR38* by binding to the ecdysone response element (EcRE) in the upstream regulatory region of this gene. The present work has implicated AHR38 in the 20E-mediated control of CM and provided new insight into mechanisms of 20E regulation of metabolism during female mosquito reproduction.

Graphical Abstract

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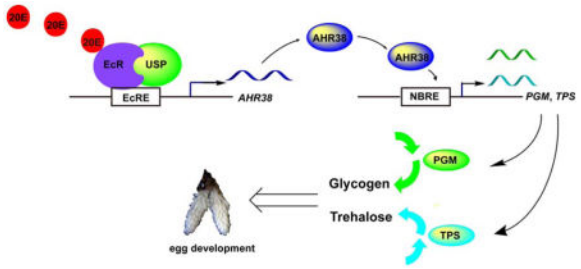

Keywords

hormone; nuclear receptor; metabolism; mosquito; disease vector

1. Introduction

Mosquito-borne diseases cause nearly a million deaths annually (Matthews, 2011). The lack of effective vaccines, fast-growing insecticide- and drug-resistance complicates this tragic situation. The yellow fever mosquito *Aedes aegypti* is a vector of several serious human diseases, such as Dengue fever, yellow fever, Chikungunya and Zika (Gloria-Soria et al., 2014; Tsetsarkin et al., 2016; Younger, 2016). Vector mosquitoes have evolved as obligatory blood feeders to utilize this food source for their rapid egg development and, as a result of this evolutionary adaptation, they are effective disease vectors. High energy requirement of a reproducing female mosquito is supported in large measure by carbohydrate metabolism (CM) (Hou et al., 2015). Therefore, a detailed understanding of CM and its regulation during mosquito reproduction could provide valuable targets for novel mosquito-control strategies.

In female mosquitoes, intake of blood activates vitellogenesis and egg development. An insect steroid hormone, 20-hydroxyecdysone (20E), is the main regulator of these reproductive processes (Hagedorn, 1989; Roy et al., 2017). Hou *et al.* (Hou et al., 2015) have reported that 20E and its receptor, ecdysone receptor (EcR), are critical regulators of CM during the post blood meal (PBM) reproductive phase in female *Ae. aegypti* mosquitoes. CM-related genes were significantly downregulated in *EcR* RNA interference (RNAi)-depleted mosquitoes. Moreover, these mosquitoes exhibited phenotypic reversal manifestations, including pronounced glycogen accumulation and reduction of CM enzyme levels. However, the molecular mechanism of 20E and EcR regulation of CM has remained unclear.

The nuclear receptor 4A (NR4A) family is evolutionarily ancient. These receptors have pleiotropic roles in regulating metabolism in mammals (Ranhotra, 2015), and the activity of this family of orphan nuclear receptors is controlled primarily at the transcription and posttranscriptional levels (Zhao and Bruemmer, 2010). Multiple extracellular signals, such as nerve growth factors (Milbrandt, 1988), inflammatory signals (Pei et al., 2005) and hormones (Pirih et al., 2005), induce NR4As. The NR4A bind to the nerve growth factor-induced protein B response element (NBRE) and activate target gene transcription (Wilson et al., 1991). The single fly ortholog of the mammalian NR4As, DHR38, regulates glycogen

storage in *Drosophila melanogaster* larvae (Ruaud et al., 2011). The NR4A ortholog AHR38 has also been identified in the mosquito *Ae. aegypti* (Zhu et al., 2000).

Here, we investigated whether AHR38 is involved in regulation of metabolism during reproduction of female mosquitoes. Indeed, we found that *AHR38* RNA interference (RNAi) depletion resulted in downregulation of the genes encoding key CM enzymes. In addition, it causes glycogen accumulation and reduction in the circulating sugar trehalose and fecundity. AHR38 transcriptionally activates the CM genes encoding phosphoglucomutase (PGM) and trehalose-6-phophate synthase (TPS), which are involved in glycogenolysis and trehalose biosynthesis. Importantly, our study has found a link between 20E and AHR38 in CM regulation. We have demonstrated that *EcR* RNAi inhibits *AHR38* gene expression. Moreover, the 20E-induced EcR-USP complex activates *AHR38* directly by binding to the ecdysone response element (EcRE) in the upstream regulatory region of this gene. This work has shown that AHR38 is involved in 20E-mediated CM. It provides new insight into 20E regulatory mechanisms of reproduction and metabolism.

2. Material and methods

2.1. Mosquito rearing

Ae. aegypti mosquito larvae were cultured at 27 °C in water supplemented with a mixture of yeast and rat chow (1:1 ratio). Adult mosquitoes were maintained at 27 °C, 86% humidity, and supplied with unlimited access to 10% (wt/vol) sucrose solution and water. All dissections were performed in *Aedes* physiological solution at room temperature (Roy et al., 2007). Four day old adult females were blood fed on white Leghorn chicken. All procedures for the use of vertebrate animals were approved by University of California, Riverside Institutional Animal Care and Use Committee.

2.2. RNA extraction and Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fat bodies using TRIzol (Invitrogen) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using the Superscript III first-strand synthesis supermix for qRT-PCR (Invitrogen). Relative transcript levels were measured in a CFX96 Thermal Cycler (Bio-Rad) by means of the iQ SYBR Grenn Supermix kit (Bio-Rad) using specific primer sets (Table 1). Each sample was performed in triplicate, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method through normalized with ribosomal protein S7 (RPS7).

2.3. RNA interference (RNAi)

Double-stranded RNA (dsRNA) was synthesized using the MEGA script RNAi Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The luciferase gene (Luc) was used to generate control its dsRNA (iLuc). A sample of 0.5 μg (0.5 μl of 1 μg/μl) dsRNA was microinjected into the thorax of female mosquitoes at 24 h PE. After 3–4 days of recovery, mosquitoes were blood-fed, and the fat body RNA was collected at 36 h PBM and subjected to qRT-PCR analysis. The primers used in the RNAi are listed in Table 1.

2.4. Glycogen and Trehalose measurements

Glycogen assays were performed using Glycogen Assay Kit (Caymon Chemical) according to the manufacturer's instructions. Female mosquito abdominal walls with adhered fat bodies were frozen and then minced into small pieces. 10–20 mg of minced tissue was homogenized in 1 ml of the assay buffer containing protease inhibitors (supplied in the kit). After centrifugation at 800×g for 10 minutes at 4°C, 10 μl supernatant was transferred to the 96-well solid plate. 50 μl of the glycogen hydrolysis enzyme (supplied in the kit) was added to sample wells, and then incubated at 37°C for 30 minutes. After incubation with glycogen fluorometric detector for 15 minutes, the fluorescence was measured by the Wallac VICTOR2 multilabel counter (PerkinElmer Life Sciences) with an excitation wavelength of 530–540 nm and an emission wavelength of 585–595 nm. The glycogen standard solution was treated the same as the samples. The glycogen concentration of the samples was calculated using the equation obtained from the linear regression of the glycogen standard curve.

For trehalose measurements, Trehalose Assay Kit (Megazyme) was used. Female mosquito whole bodies (5–10 mg) were homogenized in 1 ml of hot distilled water (~80°C). After filtering through Whatman No. 1 (9 cm) filter circle, 20 μl sample solution was transferred into a 96-well clear flat-bottom microplate. Analysis buffer (20 μl), NADP+/ATP (10 μl), and hexokinase/glucose-6-phosphate dehydrogenase (2 μl) were added into sample wells. The absorbance A1 was measured at 340 nm by Wallac VICTOR2 multilabel counter (PerkinElmer Life Sciences). After mixing with Trehalose suspension (2 μl) for 5 minutes, the absorbance A2 was measured at 340 nm. The trehalose standard solution was treated the same as the sample. Trehalose content was determined using the following formula: trehalose content (g/l) = $(A2\text{-}A1)_{Sample}/(A2\text{-}A1)_{Standard}$ × Standard trehalose content(g/l).

2.5. Cell culture and Luciferase reporter assay

Drosophila S2 cells (Invitrogen) and EcR-deficient *D. melanogaster* L57-3-11 cell line (Hu et al., 2003), obtained from Indiana University Drosophila Stock Center, were grown in Schneider's Drosophila medium (Gibco) containing 10% heat inactivated fetal bovine serum (Gibco) with supplemental Penicillin (100 units/ml)/Streptomycin (100 μg/ml) (Gibco) at 28°C in a humidified incubator.

The reporter plasmids were constructed by inserting the 5′-upstream regulatory regions of PGM (phosphoglucomutase) (3430 bp, pGL4.17-PGM), TPS (trehalose-6-phosphate synthase) (2409 bp, pGL4.17-TPS) and AHR38 (2008 bp, pGL4.17-AHR38) into the pGL4.17 vector (Promega) separately. For the NBRE-luciferase report plasmid (pGL4.17-NBRE), DNA sequences were synthesized to include seven copies of NBRE, followed by a minimal promoter from the pGL4.23 vectors. These sequences were cloned to the pGL4.17 vector. The overexpression of vectors was created by insertion of the ORF of AHR38 (Zhu et al., 2000) (AF165528.1) into the pIEx4 vector with His tag, and insertion of the ORF of EcR (Cho et al., 1995)(AAA87394.1) and USP (Kapitskaya et al., 1996) into the pAFW (Addgene Vector Database) vector with Flag tag. For the dual-luciferase reporter assay, the cells were seeded in a 24-well plate containing 450 μl medium per well, and cultured for 24 h. The reporter (100 ng per well) and overexpression (50–150 ng per well) plasmids were

co-transfected with a pCopia plasmid (15 ng per well) encoding *Renilla* luciferase using FuGENE HD Transfection Reagent (Promega). Then, the cells were processed with the Dual-Luciferase reporter assay system (Promega) at 48 h after transfection. Relative luciferase activity was measured using the GloMax 96 microplate luminometer (Promega), and data were normalized against *Renilla* luciferase activity.

2.6. Western blot analysis

The protein levels of AHR38, EcR and USP were identified by means of immunoblot. After treatment in a passive lysis buffer, 10 μg protein was separated on 4–12% Tris-glycine gels (Invitrogen) and then transferred to PVDF membranes (Invitrogen). The following antibodies were used: anti-His antiserum (Abcam, ab18184) for AHR38-His; anti-Flag antiserum (Abcam, ab18230) for EcR-Flag and USP-Flag; β-actin monoclonal antibody (Sigma, A5441) for actin.

2.7. In vitro fat body culture

The *in vitro* fat body culture was performed as previously described (Roy et al., 2007). Previtellogenic female mosquito abdominal walls with adhered fat body tissue were incubated in a complete culture medium supplemented with a complete set of amino acids and 20E (10^{-6}M) for 6 h. Total RNA was then extracted, and transcript abundance was analyzed using qRT-PCR.

2.8. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously (Wang et al., 1998). Oligonucleotides used in EMSA were: *AHR38* putative EcRE probe 5′-ctagcttAGGTTAATCGATTcaagtgagga-3′; mutant probe 5′-ctagcttCCAGATCGATTGAcaagtgagga-3′.

3. Results

3.1. AHR38 transcription is coordinated with the expression pattern of CM genes

We conducted a time-course transcription analysis of the *AHR38* gene in the fat body of female *Ae. aegypti* mosquitoes. qRT-PCR was used to monitor the abundance of the *AHR38* and selected CM gene transcripts at eight time points between 72 h post eclosion (PE) and 72 h post blood meal (PBM) during the first reproductive cycle. *AHR38* mRNA peaked in abundance 36 h PBM and then declined gradually (Fig. 1A). The genes encoding key enzymes for CM—including two that catalyze glycogenolysis (Gly, glycogen phosphorylase; PGM), one that catalyzes trehalose synthesis (TPS), and three rate-limiting enzymes of glycolysis (HEX, hexokinase; PFK, phosphofructokinase; PYK, pyruvate kinase) (Fig. 1B)—exhibited an upward trend after blood feeding and reached their maximal expression by 36 h PBM (Fig. 1A), which was similar to the *AHR38* expression kinetics.

3.2. Silencing of AHR38 by RNAi results in retardation of egg development

AHR38 was silenced by injecting dsRNA into the female mosquitoes at 24 h PE. The knockdown efficiency was evaluated using qRT-PCR at 36 h PBM. The results showed that after *AHR38* RNAi depletion, the transcript level decreased to 42% compared with

Luciferase RNAi (iLuc) control (Fig. 2A). We then analyzed the effect of *AHR38* RNAi depletion on ovarian development in female mosquitoes. The *AHR38* RNAi-depleted, blood-fed females failed to fully mature ovaries (Fig. 2B). The length of ovarian follicles measured at 36 h PMB was reduced by 23.1% (253.6 μm on average) compared with those in the WT or iLuc-controls (Fig. 2C). In addition, *AHR38* RNAi depletion resulted in a dramatically reduced egg deposition. The females injected with *AHR38* dsRNA laid an average of only 62.3 eggs per mosquito, whereas the WT and iLuc controls laid 121.5 and 114.5 eggs per female, respectively (Fig. 2D).

3.3. AHR38 RNAi silencing affects the levels of storage and circulating sugars

We measured levels of stored and circulating sugars after *AHR38* RNAi knockdown. As shown in Fig 3A, *AHR38* dsRNA treatment resulted in a significant increase in glycogen levels at 36 h PBM. At the same time, trehalose, the main circulating hemolymph sugar in insects, declined in abundance in *AHR38*-depleted female mosquitoes. These data imply that *AHR38* knockdown impaired the utilization of glycogen and stability of circulating sugar levels, thus negatively affecting reproduction.

3.4. Transcriptional activation PGM and TPS by AHR38

Due to the AHR38 effect on metabolism of storage and circulating sugars, we selected genes encoding key enzymes responsible for these metabolic steps (*PGM* and *TPS*) as possible targets of AHR38 control. Unlike the iLuc control, both *PGM* and *TPS* transcripts declined at 36 h PBM after *AHR38* RNAi silencing (Fig. 3C and 3D). This suggests that AHR38 activates the transcription of the *PGM* and *TPS* genes.

We hypothesized that being a transcription factor AR38 may exert its effect on *PGM* and *TPS* expression through a promoter interaction. In *Drosophila*, DHR38 binds specifically to the NBRE, which includes one AGGTCA half-site and two adenosines upstream from the half-site (Wilson et al., 1991). To investigate whether AHR38 could bind specifically to the NBRE, a luciferase reporter pGL4.17-NBRE was constructed using seven tandem NBRE copies (Fig. 4A). pGL4.17-NBRE was activated by the overexpression of the AHR38 expression vector in S2 cells in a dose-dependent manner (Fig. 4C). Transfection of 100 ng AHR38 expression vector resulted in an 8.5-fold activation of the NBRE-luciferase reporter (Fig. 4C). Sequence analysis of *PGM* and *TPS* showed the presence of potential NBREs in 5′-upstream regulatory regions of *PGM* and *TPS* (Fig. 4B). Dual-luciferase reporter assay showed the ability of an *in vitro* translated AHR38 protein to transcriptionally activate *PGM* (Fig. 4D) and *TPS* (Fig. 4E) gene promoters.

3.5. AHR38 RNAi silencing blocks 20E activation of PGM and TPS expression

In mosquitoes, expression of genes involved in CM is activated after a blood meal through the synergistic action of a steroid hormone and the amino acid/target of rapamycin (TOR) signaling pathway (Hou et al., 2015). We considered the possibility that AHR38 serves as an intermediate factor of the 20E action on CM genes. To test this hypothesis, we used the *in vitro* fat body culture assay in which fat body tissue isolated from mosquitoes at 72h PE was incubated in the presence of 20E and AAs. After *AHR38* RNAi knockdown (Fig. 5A), the incubation of the fat body in the medium containing AAs and 20E could not elevate the

transcript abundance of *PGM* (Fig. 5B) or *TPS* (Fig. 5C). This data indicates that AHR38 mediates the regulation of the CM genes by 20E in the presence of AAs.

3.6. 20E up-regulates AHR38 via direct binding of EcR to its promoter

Because AHR38 RNAi depletion blocks the effect of 20E on expression of *PGM* and *TPS*, we hypothesized that this nuclear receptor might be a downstream mediator of the 20E control of CM gene expression. To investigate this hypothesis, we checked the effect of 20E on *AHR38* expression. The result of qRT-PCR showed that 20E or AAs alone enhanced the expression of AHR38 by only 2-fold. However, the 20E and AA combination increased the expression of AHR38 by 10-fold after the challenge (Fig. 6A). These results indicate that *AHR38* was induced by 20E, particularly in the presence of AAs.

As the major hormone governing PBM reproductive events in female mosquitoes, 20E plays its roles via EcR, which together with its heterodimeric partner USP forms the functional 20E receptor (Thomas et al., 1993). To determine whether EcR mediates the 20E induction of *AHR38*, we knocked down *EcR* using dsRNA to its common region (iEcR) in female mosquitoes at 24 h PE (Fig. 6B) and analyzed *AHR38* transcript levels at 36 h PBM. *AHR38* expression was significantly suppressed as a result of EcR RNAi silencing, indicating that EcR positively affects expression of the *AHR38* gene (Fig. 6C).

The observation that *AHR38* expression is upregulated by EcR raises the question of whether the effect of EcR on this gene is direct via interaction with its promoter or indirect via intermediate factors. We first carried out the dual-luciferase reporter assay. For this test, we over-expressed AaEcR and AaUSP in EcR-deficient L57-3-11 cells in the presence of the AHR38 promoter/reporter construct containing putative Ecdysone Receptor binding site (EcRE). Western blotting was used to identify the expression levels of EcR and USP with anti-Flag antibody. Dual-luciferase reporter assay shows the transcriptional activation *AHR38* promoter/reporter by EcR/USP in L57-3-11 cells in the presence of 20E (Fig. 7A). To determine whether the EcR-USP complex physically interacts with the *AHR38* promoter, we performed Electrophoretic Gel Mobility Assay (EMSA) experiments using ^{32}P-labeled oligonucleotide fragment containing the *AHR38* gene putative EcRE sequence (Fig. 7B). A dense band was observed in nuclear extracts from L57-3-11 cells with expressed EcR-Flag and USP-Flag in the presence of 20E. The specificity of the protein-DNA interaction was confirmed by a competition with an excess of the unlabeled specific probe. The band was dramatically diminished after nuclear extracts were pre-incubated with anti-Flag antibody, indicating the binding of antibody (anti-Flag) and antigen (EcR-Flag/USP-Flag) interfered the formation of protein-DNA complex (EcR-Flag/USP-Flag and the probe), thus, there was no super-shift was observed. The interaction disappears after the probe was mutated (Fig. 7B, right panel). Thus, these data strongly indicate that the EcR/USP complex directly binds to the EcRE sequence located at the *AHR38* promoter.

4. Discussion

Previously, we have identified 20E and its receptor EcR to be critical regulators of CM during the PBM reproductive phase in female *Ae. aegypti* mosquitoes (Hou et al., 2015). Here, we have shown that AHR38, a member of the NR4A subfamily of orphan nuclear

receptors, is a positive regulator of CM genes during mosquito reproduction. It does so via a transcriptional activation binding to the NBRE-like sequence in promoters of CM genes, such as *PGM* and *TPS*. The most important finding of this work is establishing a regulatory link between AHR38 and the ecdysteroid pathway in CM control. *AHR38* RNAi phenocopies the effect of *EcR* RNAi with respect to CM metabolism; it causes downregulation of CM genes and accumulation of glycogen. Moreover, *AHR38* is upregulated by 20E and EcR, and EcR affects this nuclear receptor expression through an interaction with its promoter. Thus, AHR38 serves as the mediator of 20E/EcR regulation of glycogenolysis and glycolysis during *Ae. aegypti* female reproduction.

Hormone receptor HR38 is an ortholog of the NR4A subfamily of orphan nuclear receptors that belongs to the larger nuclear receptors (NRs) superfamily of eukaryotic transcription factors. Functional studies have revealed that the NR4A receptors play an important role in maintaining metabolic homeostasis by regulating carbohydrate metabolism (Hummasti and Tontonoz, 2008). *DHR38*, the single fly ortholog of the mammalian NR4A receptors, controls glycogen storage during the larval stages of *D. melanogaster* (Ruaud et al., 2011). Consistent with the observation, we report here that *AHR38*, the mosquito homolog of NR4A, regulates the glycogen storage and the hemolymph trehalose level. Interestingly, *DHR38* mutants display reduced levels of glycogen in the muscle but not in tissues like the fat body or the gut (Ruaud et al., 2011). We have observed that *AHR38* RNAi silencing increased the glycogen levels in the mosquito fat body. However, we have not investigated the glycogen distribution in other mosquito tissues.

In mammalians, all three NR4A proteins modulate target gene transcription by binding to the NBRE sequence (AAAGGTCA) (Wilson et al., 1991). Our dual-luciferase reporter assay showed that AHR38 also exerts its function via interaction with the promoters of *PGM* and *TPS* containing NBRE-like sequences and thereby induces their transcription.

In contrast to classical ligand-activated nuclear receptors, the NR4A receptors are ligand-independent nuclear receptors as proved by crystallography studies showing the ligand-binding pocket is filled with bulky hydrophobic groups (Wang et al., 2003). Activity of this family of nuclear receptors is controlled primarily at the level of gene expression. NR4As are induced by multiple extracellular signals, including growth factors, cytokines, neurotransmitters and peptide hormones, in a cell type–specific manner (Pei et al., 2006). In *Drosophila* larvae, the *DHR38* expression is activated by nutritional signaling (Ruaud et al., 2011). However, the basis of this nutritional signaling remains unclear. Here, our *in vitro* fat body culture assay showed that steroid hormone 20E induced the expression of *AHR38*, especially in the presence of AAs, which is consistent with the transcription pattern of *AHR38 in vivo*. qRT-PCR analysis has demonstrated that the *AHR38* mRNA level increased during the PBM stage, during which the ingestion of blood results in a release of a large quantity of AAs. Thus, in addition to the hormonal 20/EcR signaling the AHR38 expression may also be regulated by a nutritional AA-Target of Rapamacin (TOR) pathway. Further studies are necessary to verify this suggestion. Synergistic action of AA/TOR and 20E/EcR signaling has been demonstrated in expression regulation of numerous genes in female mosquitoes after blood feeding (Hansen et al., 2004; Roy et al., 2015).

20E is the major regulator of the PBM phase of the female mosquito reproductive cycle, and its action is mediated by EcR (Raikhel et al., 2002; Roy et al., 2018). EcR RNAi silencing has been reported in mosquitoes to result in reduced transcript levels of the CM genes (Hou et al., 2015). AHR38 has been implicated in 20E-regulated metabolic control based on our observation that *AHR38* RNAi knockdown blocks the 20E-mediated induction of the CM genes. Moreover, experiments have shown that the EcR/USP complex binds to the EcRE located at the AHR38 promoters of CM genes and activates their transcription. Direct binding of EcR/USP to target genes has been previously reported and is particularly well studied for mosquito vitellogenin (*Vg*) gene (Zhu et al., 2006). However, the interaction of EcR/USP with early gene transcription factors E74, E75 and *broad* on the *Vg* promoter is required to attain maximal expression level of this gene (Cruz et al., 2012; Sun et al., 2005; Zhu et al., 2007). Future studies should clarify whether early genes are involved in EcR/USP transcriptional control of the *AHR38* gene. In conclusion, our study has implicated AHR38 to be a critical mediator of 20E/EcR pathway in the regulation of CM genes during the PBM phase of the gonadotrophic cycle in female mosquitoes.

Acknowledgments

This work was supported by NIH/NIAID Award 5RO1 AI036959 to ASR and China Scholarship Council of Chinese Ministry of Education Scholar Fellowship 201506225080 to DD.

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Highlights

- Carbohydrate metabolism (CM) is essential for supplying energy in the mosquito *Aedes aegypti*.
- The steroid hormone 20-hydroxyecdysone (20E)is a n important regulator of CM.
- The NR4A nuclear receptor AHR38 mediates 20E control of CM genes by binding to their promoters.
- In turn, ecdysone receptor activates *AHR38* via interaction with its promoter.

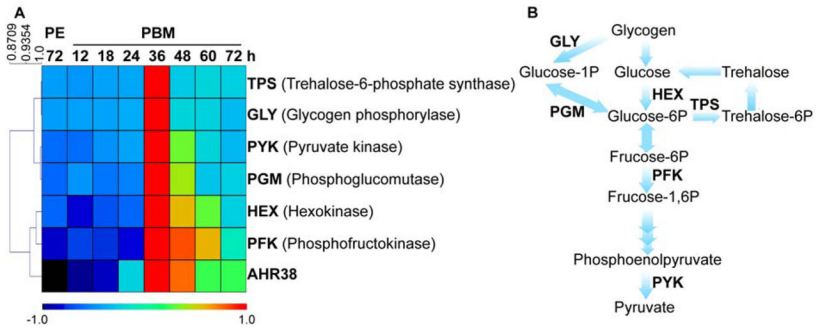

Fig. 1.

AHR38 mRNA peaked in abundance at 36 h post blood meal (PBM), which is similar to that of the genes encoding key enzymes for carbohydrate metabolism (CM). (A) Heatmap representing the qRT-PCR-based relative expression pattern of *AHR38* and key CM genes in the female mosquito fat body. Relative expression was analyzed at the following time points: 72 h post-eclosion (PE), and 12, 18, 24, 36, 48, 60, and 72 h PBM. The transcript abundance was based on the $2^{-\Delta\Delta CT}$ method in the presence of *RPS7* gene. The color code indicates the fold change of transcript abundance in the form of a logarithm. Each transcript abundance value was normalized. The dendrograms were constructed based on Pearson complete correlation. (B) A schematic diagram showing the CM pathway with key CM enzymes involved in glycogenolysis and glycolysis.

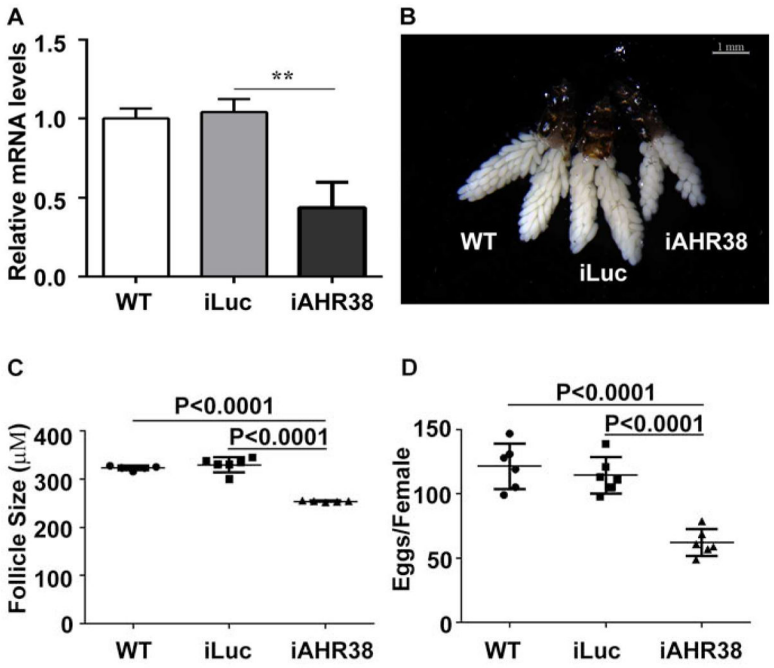

Fig. 2.

AHR38 RNA interference (RNAi) knockdown results in the reduction of ovarian follicle length and the number of laid eggs. *AHR38* dsRNA (0.5 μg) was injected into the thorax of female mosquitoes at 24 h PE; the control group was treated with dsRNA of luciferase (iLuc). (A) qRT-PCR analyses show the efficiency of *AHR38* knockdown. (B–D) The phenotype of *AHR38* knockdown: ovaries (B), average follicle size (C) and egg number (D) from WT, iLuc and iAHR38 female mosquitoes at 36 h PBM. Data represent three biological replicates with three technical replicates and are shown as mean ± SEM. Asterisks indicate significant differences (Student's *t* test: **, $p<0.01$).

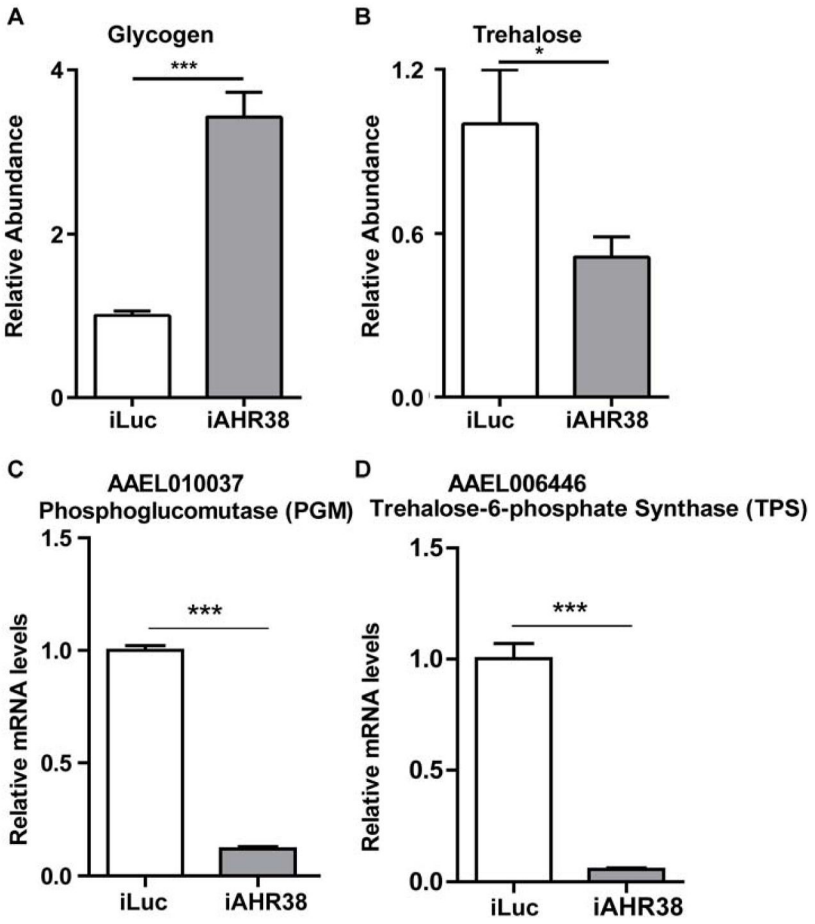

Fig. 3.

AHR38 RNAi silencing results in changes in the levels of glycogen and trehalose as well as reduction in transcript levels of *PGM* and *TPS. AHR38* was knocked down by injecting dsRNA (0.5 μg) at 24 h PE. (A) Glycogen assays were performed using the Glycogen Assay Kit (Caymon Chemical) at 36 h PBM. Fluorescence was measured with an excitation wavelength of 530–540 nm and an emission wavelength of 585–595 nm. (B) Endogenous levels of the circulating sugar, trehalose, were measured using Trehalose Assay Kit (Megazyme). The absorbance was read at 340 nm. qRT-PCR analysis of the transcription of *PGM* (C) and *TPS* (D) after AHR38 knockdown. The results were normalized by iLuc group. Data represent three biological replicates with technical replicates and are illustrated as mean ± SEM. Asterisks indicate significant differences (Student's *t* test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

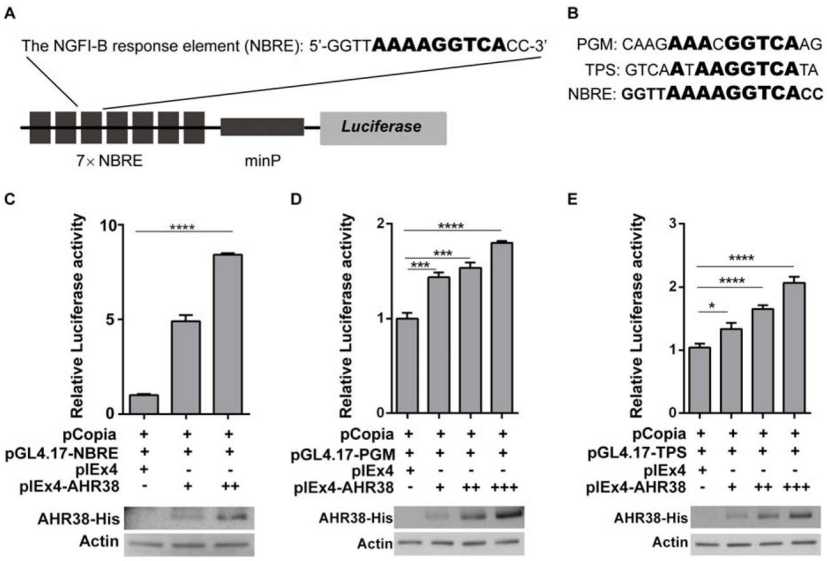

Fig. 4.

Transcriptional activation of the NBRE-containing luciferase reporter, *PGM* and *TPS* by AHR38 in *Drosophila* S2 cells. (A) A luciferase reporter pGL4.17-NBRE construct carrying seven copies of NBRE and the minimal promoter sequence derived from the pGL4.23 vector that was used in (C). Similar constructs with either *PGM* or *TPS* sequences shown in (B) were used in (D) and (E). (B) Sequence comparison of the NBRE core sequence with those from the 5′-upstream regulatory regions of *PGM* and *TPS*, as indicated by the capital bold letters. Dual-luciferase reporter assay analysis of the activation of pGL4.17-NBRE (*C*), pGL4.17-PGM (D), and pGL4.17-TPS (E) by AHR38. S2 cells were co-transfected by luciferase reporter vectors (pGL4.17-NBRE, pGL4.17-PGM, pGL4.17-TPS, 100 ng) and increasing amounts of AHR38-His expressing plasmid (pIEx4-AHR38, "+"=50 ng). Relative luciferase activity was normalized to *Renilla* luciferase activity and plotted as mean ± SD (n=3). Western blots showing the expression of AHR38-His in S2 cells with anti-His antibody. Actin was used as the loading control.

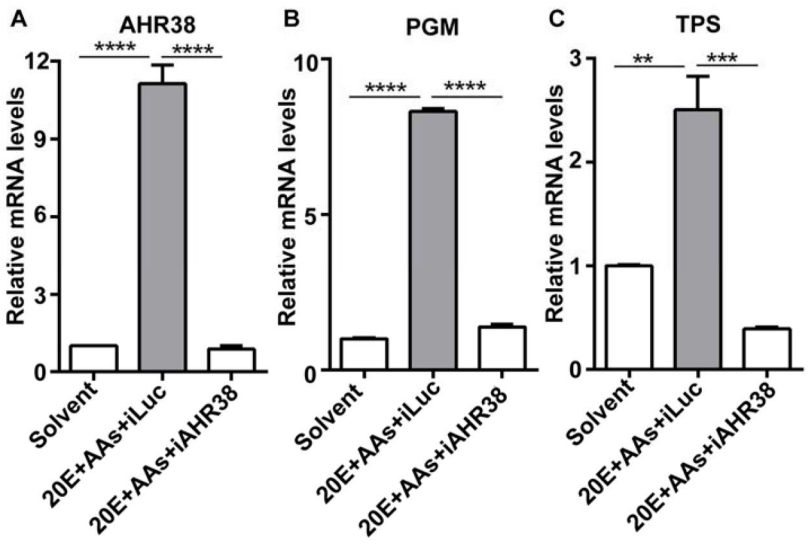

Fig. 5.

AHR38 depletion blocks 20E induction of *PGM* and *TPS* expression in the presence of amino acids (AAs). After the *AHR38* was knocked down by injecting dsRNA of *AHR38* at 24 h PE, the fat body tissue was isolated at 72 h PE and cultured *in vitro*. 20E (10^{-6}M) was added to the culture medium supplemented with a complete set of AAs for 6 h. (A) Efficiency of RNAi was checked via qRT-PCR. This analysis shows that the transcription of *PGM* (B) and *TPS* (C) could not be induced by 20E after AHR38 had been silenced. Data represent three biological replicates with three technical replicates, illustrated as mean ± SEM. Asterisks indicate significant differences (Student's *t* test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

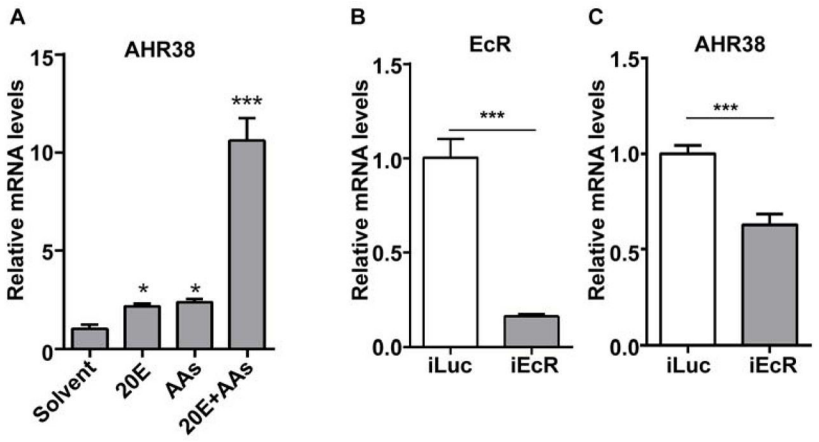

Fig. 6.

20E induced *AHR38* via EcR. (A) Fat body tissue isolated from mosquitoes at 72 h PE was incubated in the presence of AAs and/or 20E. qRT-PCR results indicate that 20E induced the transcript of *AHR38* particularly in the presence of AAs. (B) Knockdown of EcR by injecting dsRNA at PE 24 h. The efficiency of RNAi was checked at 36 h PBM via qRT-PCR. (C) EcR RNAi silencing suppressed the transcript of *AHR38*.

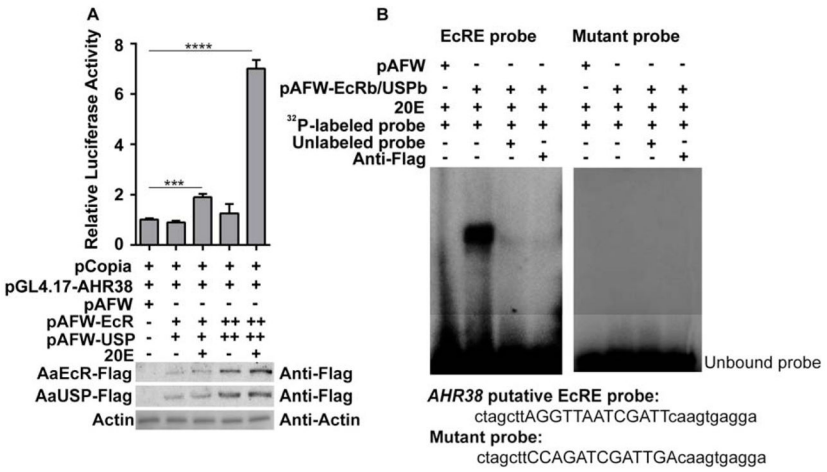

Figure 7.

Transcriptional activation of the *AHR38* gene via EcR binding to its promoter. (A) Dual-luciferase reporter assay shows the transcriptional activation AHR38 promoter by EcR/USP in L57-3-11 cells in the presence of 20E. L57-3-11 cells were co-transfected by the reporter vector pGL4.17-AHR38 (100 ng), the overexpression plasmids (pAFW-EcR and pAFW-USP, 100 ng), and the pCopia plasmid (15 ng). The relative luciferase activity was analyzed at 48 h after transfection. Western blotting was used to check the expression levels of EcR and USP with anti-flag antibody. (B) EMSA using the ^{32}P-labeled probe containing *AHR38* putative EcRE sequence and nuclear protein extracts from L57-3-11 cells with expressed EcR-Flag and USP-Flag and treated with 10^{-6} M 20E.

Table 1

Oligonucleotide sequences of PCR primers

Primer name	Oligonucleotide sequence (5′-3′)
qRT-PCR	
GLYQRTF	GCCTGGATATGGCAACAACT
GLYQRTR	ACACGGGAAATGTTTTCAGC
PGMQRTF	AAGGACGGAGTGGTTCACAC
PGMQRTR	ATGTCGTAATCTCCGTTCCG
TPSQRTF	GAAACGTGTGCTCAGAACCA
TPSQRTR	TTTGCAGCATGCTTACGTTC
HEXQRTF	CTAGATCTGGGCGGTACCAA
HEXQRTR	GTCCCACTGCCAAGCATAAT
PFKQRTF	GCCGGTCAAAGATTGAACAT
PFKQRTR	AGCACCGTAATCCTGGTGTC
PYKQRTF	CGTTGAACAGGGTGTTGATG
PYKQRTR	CCTGCTGGTTCTCGATCTTC
AHR38QRTF	GCGGTAAGTCAGCCCAGAA
AHR38QRTR	GGCGGAAGGGAATAGGAAT
EcRQRTF	AAGCGAGGTTATGATGTTGCG
EcRQRTR	CAGCAGGTCCTCTATCGTGTCC
RPS7QRTF	TCAGTGTACAAGAAGCTGACCGGA
RPS7QRTR	TTCCGCGCGCGCTCACTTATTAGATT
dsRNA synthesis	
iAHR38F	TAATACGACTCACTATAGGGATGGTCAAGGAAGTTGTCCG
iAHR38R	TAATACGACTCACTATAGGGAGCGTTCTGTCACCAAGGTT
IEcRF	TAATACGACTCACTATAGGGCGGAGCGTCACCAAGAATG
IEcRR	TAATACGACTCACTATAGGGTGGCGAACTCCACGATTAG
ILucF	TAATACGACTCACTATAGGGCCTGGATCACTACAAGTACCTCA
ILucR	TAATACGACTCACTATAGGGCGACAATAGCGTTGGAAAA
Dual-luciferase report assay	
pGL4.17-NBREF	TACTCACTCGAGGGTTAAAAGGTCACCAGG
pGL4.17-NBRER	TACTCAAAGCTTAAGCTGGAAGTCGAGCTTC
pGL4.17-PGMF	GTGTGAGCTCGAGTTGCTCAGAGTTATGGC
pGL4.17-PGMFR	TCCTCTCGAG CTGTGCTCTAGAGGTGTTCC
pGL4.17-TPSF	GTGTGAGCTCCTGGAACAAGTGTAGACCTGAAG
pGL4.17-TPSR	TCCAAGCTTTGTGAACTTTTCTTCTGGTTC
pGL4.17-AHR38F	TACTCAGGTACCACTTTGGCTCGTTTGTTTA
pGL4.17-AHR38R	TACTCAGAGCTCGAAGCAATCTTCGTCCAT
AHR38-pIEx4F	GTGTGAGCTCAACTCTTTTAGTTCAGTTTC
AHR38-pIEx4R	CTCTGGTACCGAACGGCAAACTGGCGAG