1-29-2014

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Recommended Citation

Perez-Hedo M, Rivera-Perez C, Noriega FG (2014) Starvation Increases Insulin Sensitivity and Reduces Juvenile Hormone Synthesis in Mosquitoes. PLoS ONE 9(1): e86183. doi:10.1371/journal.pone.0086183

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Starvation Increases Insulin Sensitivity and Reduces Juvenile Hormone Synthesis in Mosquitoes

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Abstract

Background: The interactions between the insulin signaling pathway (ISP) and juvenile hormone (JH) controlling reproductive trade-offs are well documented in insects. JH and insulin regulate reproductive output in mosquitoes; both hormones are involved in a complex regulatory network, in which they influence each other and in which the mosquito's nutritional status is a crucial determinant of the network's output. Previous studies reported that the insulin-TOR (target of rapamycin) signaling pathway is involved in the nutritional regulation of JH synthesis in female mosquitoes. The present studies further investigate the regulatory circuitry that controls both JH synthesis and reproductive output in response to nutrient availability.

Methods: We used a combination of diet restriction, RNA interference (RNAi) and insulin treatments to modify insulin signaling and study the cross-talk between insulin and JH in response to starvation. JH synthesis was analyzed using a newly developed assay utilizing fluorescent tags.

Conclusions: Our results reveal that starvation decreased JH synthesis via a decrease in insulin signaling in the corpora allata (CA). Paradoxically, starvation-induced up regulation of insulin receptor transcripts and therefore “primed” the gland to respond rapidly to increases in insulin levels. During this response to starvation the synthetic potential of the CA remained unaffected, and the gland rapidly and efficiently responded to insulin stimulation by increasing JH synthesis to rates similar to those of CA from non-starved females.

Introduction

The correct allocation of nutrients between conflicting needs such as reproduction, growth, maturation or flight is a vital component of an insect's life-history strategy [1]. Juvenile hormone [JH] is a remarkably versatile molecule with major effects on various aspects of development and life history in insects, including metamorphosis, behavior, reproduction, diapause, stress resistance and aging [2]. The insulin signaling pathways [ISP] is an evolutionary conserved systemic nutritional sensor that plays a central role in the transduction of nutritional signals that regulate cell growth and metabolism [3,4]. JH and insulin are involved in a complex regulatory network, in which they influence each other and in which the insect’s nutritional status is a crucial determinant of the network's output [5].

The reproductive cycle of female mosquitoes proceeds through a previtellogenic stage (PVGS), an ovarian resting stage (ORS) and a vitellogenic stage (VGS) [6]. In Aedes aegypti, JH is a key mediator of trade-offs between survival and reproduction during the PVGS and ORS [7,8]. Regardless of the nutritional status of a female mosquito, topical application of JH will override other signaling pathways and stimulate oogenesis even in isolated abdomens [9,7]. In addition, increased insulin production and signaling, in response to a positive nutritional status, tends to stimulate egg production in mosquitoes [10].

How these two hormonal signaling pathways (ISP and JH) interact to regulate all the physiological functions necessary for egg production is not totally clear. JH titers in female adult mosquitoes are mainly determined by the rate at which the corpora allata (CA) synthesize JH [11]. Changes in JH synthesis in female adult A. aegypti mosquito are very dynamic and nutrition-dependent [12]. Previous studies reported that the insulin/target of rapamycin (TOR) pathway is involved in the nutritional regulation of JH synthesis in female mosquitoes [13]. Application of bovine insulin on the mosquito CA-CC incubated in vitro caused a strong and fast stimulation on JH synthesis; while systemic depletion of TOR by RNAi and rapamycin administration had inhibitory effects on JH synthesis [13]. In addition, reducing insulin signaling with PI3K inhibitors rapidly reduced JH biosynthetic enzyme transcripts; validating that a transcriptional regulation of the genes encoding JH biosynthetic enzymes is at least partially responsible for the dynamic changes of JH biosynthesis in mosquitoes [13].

Here diet restriction, in vivo depletion of INSr and FOXO using RNA interference (RNAi) and insulin treatments were used to modify insulin signaling and study the cross-talk between insulin and JH in response to starvation. Our results reveal that starvation...
decreased JH synthesis via a decrease in insulin signaling in the CA. Starvation-induced up regulation of the insulin receptor increased CA insulin sensitivity and might “prime” the gland to respond rapidly to increases in insulin levels after feeding resumption. During this response to starvation the synthetic potential of the CA remained unaffected, and the gland rapidly and efficiently responded to insulin stimulation by increasing JH synthesis to rates similar to those of CA from non-starved females.

Materials and Methods

Insects

*Anopheles aegypti* of the Rockefeller strain were reared at 28°C and 80% relative humidity under a photoperiod of 16 h light: 8 h dark. Female were offered a cotton pad soaked in 20% sucrose solution for the first 24 h after adult emergence. Afterwards, one-day-old females were separated into two experimental groups: starved females were offered water, and sucrose-fed females were maintained on a 20% sucrose solution. All experiments were performed with 4-day old virgin females.

To test the effect of starvation we used four days old females because our RNAi protocol requires waiting a 4-day period after injection to be effective [13]. Starving newly emerged females for 4 days resulted in an elevated mortality. Feeding females on a high sugar meal (20% sucrose) for 24 h before starving them for 3 days improved survival to almost 100%. In the rest of the manuscript we will call “sugar-fed” to those females fed 20% sugar for 4 days and “starved” or “water-fed” to those fed water from day 2 to 4.

Lipid assays

Lipids were quantified in mosquitoes using a triglyceride quantification kit (K622-100, Biovision, Mountain View, CA) as previously described [8].

Assessment of in vitro JH production

Corpora allata-corpora cardiaca complexes (CA-CC) attached to the head capsule were isolated from adult females as previously described [11]. JH synthesis was evaluated on CA-CC incubated at 32°C in M-199 medium (Lavallette, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and methionine (50 mM). Bovine insulin was purchased from commercial sources (Boca Raton, FL), dissolved in HCl and used at 17 μM, a concentration previously established to be optimal [13].

Quantification of JH synthesis

The amount of JH synthesized by CA-CC complexes in vitro was quantified using high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) [14]. The assay is based on the derivatization of JH III with a fluorescent tag with subsequent analysis by reverse phase HPLC-FD.

RNA extraction, reverse transcription and real-time PCR

Total RNA was isolated using RNA-binding glass powder as previously described [15]. Contaminating genomic DNA was removed using Turbo DNA-free DNase (Ambion, Austin, TX, USA). Reverse transcription was carried out using an oligoDT priming method and SuperScript® III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA). qPCR was performed with anApplied Biosystems7300 Real Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were run in triplicate using 1 μl of cDNA per reaction in a 20 μl volume according to manufacturer recommendations for Custom TaqMan® Gene Expression Assays.
synthesis we studied its effect on the expression of juvenile hormone acid methyltransferase (JHAMT), a key JH biosynthetic enzyme. We observed a statistically significant reduction in JHAMT transcript levels in the CA of starved adult female mosquitoes (Fig. 2B).

**Starvation modulates the expression of insulin/TOR pathway genes**

To dissect the transcriptional regulatory circuitry of the insulin signaling cascade in the CA in response to starvation, transcript levels for 4 key genes were analyzed in the CA of 4 days old adult females fed sugar or water (Fig. 3). Transcript levels for the insulin receptor (INSr), the Forkhead-box-binding protein (FOXO) and the translation initiation inhibitor eIF4E-binding protein (4EBP) were significantly increased in the CA of starved females. Transcripts for the target of rapamycin (TOR) protein were significantly decreased in the CA of starved females.

**In vivo depletion of insulin receptor or FOXO modifies JH synthesis**

To further understand the role of insulin pathway components on JH synthesis we analyzed the effect of *in vivo* depletion of INSr and FOXO. Injection of dsINSr resulted in a significant reduction of INSr mRNA when compared with dsYFP treated in both starved (55%) and sugar-fed adult females (58%), without reducing FOXO’s transcript levels (Fig. 4A). In contrast, injection of dsFOXO resulted in significant reductions of both FOXO (82–85%) and INSr mRNAs (55–60%) in starved and sugar-fed adult females when compared with dsYFP treated controls (Fig. 4B).
Systemic depletion of FOXO mRNA caused a significant reduction of JH synthesis in sugar-fed and starved females (Fig. 5). On the contrary, depletion of INSr mRNA had opposite effects depending on the nutritional status of mosquitoes; it decreased JH synthesis in sugar-fed females and increased JH synthesis in starved females (Fig. 5).

**Starvation increases CA insulin sensitivity**

To test if the nutritional status modifies the responsiveness of CA to insulin stimulation in vitro, CAs were dissected from 4-day old starved and sugar-fed females and cultured for 4 h in the presence of bovine insulin (17 μM). JH synthesis was significantly stimulated by insulin in glands dissected from starved females; while CA dissected from sugar-fed females did not respond to insulin stimulation (Fig. 6). The addition of insulin increased JH...
synthesis by CA of starved females to levels equivalent to those produced by glands of sugar-fed females.

Discussion

Starvation decreases JH synthesis and reduces nutrient allocation into the ovaries

Reproductive trade-offs in adult insects occur mostly through the process of oosorption. By resorbing excess reproductive tissues, insects can adjust previous allocation decisions by redirecting resources away from reproduction in favor of competing physiological activities [16,17]. A substantial number of studies have shown that sugar feeding is critical for building energy reserves and improving egg production in a female mosquito [18,19]. Deprivation of a sugar meal had dramatic effects on nutrient stores; starvation for 3 days resulted in a 10 fold decrease in total lipid reserves. We have previously described that similar starvation conditions resulted in a 4 fold decrease in oocyte lipid content and a 4 fold increase in the rate of resorption [8]; however application of methoprene (a JH analog) to starved females increased stored ovarian lipids and decreased resorption [8].

On the other hand, lipids reserves increased more than 2-fold by feeding 20% sugar for the first 24 h after emergence, indicating that a sugar meal could rapidly augment lipid stores. It is important for a female mosquito to rapidly and effectively detect these dynamic changes in nutrient reserves and correct nutrient allocations. The interactions between the ISP and JH controlling reproductive trade-offs are well documented in insects [5]. The activity of the ISP is directly modulated by the nutritional and feeding state [20]. The A. aegypti genome encodes eight insulin-like peptides (ILPs), with three of them (ILP1, ILP3 and ILP8) specifically expressed in brains of adult females [21]. To modulate JH synthesis, the ILPs could either be directly delivered to the CA by projections of the corpora cardiaca (CC) or they are received from the circulating hemolymph. Transcript levels for several A. aegypti ILPs show age dependent and diet dependent changes in female mosquitoes [22]. While JH is the gonadotrophic hormone responsible for primarily controlling nutrient allocation into the ovary [7,8], the current findings establish that the starvation-induced reduction in JH biosynthesis is the result of a decrease in insulin signaling and it can be totally reversed by supplying exogenous insulin.

Starvation decreases insulin signaling and increases insulin sensitivity without affecting CA competence to synthesize JH

The TOR and ILP signaling pathways are considered as nutritional sensors at the cellular and systemic level, respectively. We have previously established that application of bovine insulin on the mosquito CA incubated in vitro caused a strong and fast stimulation of JH synthesis [13]. In addition, either systemic depletion of TOR by RNAi or rapamycin administration had inhibitory effects on JH synthesis [13].Furthermore, rapamycin impaired insulin stimulation of JH synthesis in the CA of mosquitoes, confirming that TOR activity is critical for the transduction of insulin signaling [13]. Our new results provide evidence that starvation decreases insulin signaling in the CA and reduces JH synthesis. We detected significant increases in CA transcript levels of some key components of the ISP, such as FOXO, 4E-BP and INSr that are well known markers for the starvation response [23]. FOXO proteins are indispensable in the response to starvation, since they promote conservation of energy and catabolism. FOXO is a major coordinator of the transcriptional response to nutrients downstream of insulin; remarkably, 99% nutrient-responsive genes were regulated by activated FOXO in Drosophila [24]. Among those critical genes are 4E-BP and INSr. We observed a transcriptional activation of the translational repressor 4E-BP in the CA. A transcriptional and post-transla-
tional regulation of this TOR effector, finely tuned to the nutritional requirements, was previously described in the fat body of female mosquitoes [25].

The insulin receptor itself is a target gene for FOXO regulation in Drosophila and mammals [23], revealing a novel feedback mechanism in the ISP. In this way the insulin receptor can regulate its own synthesis in response to nutrient availability through the modulation of FOXO activity. When nutrients are abundant, FOXO is turned off and downregulation of insulin receptor occurs. However, when nutrients become limited, insulin receptor synthesis becomes upregulated by FOXO and a higher density of receptor molecules accumulate in the cell membrane. The cells thus become “primed” and highly sensitized to respond rapidly to changes in insulin, which signal changes in nutrients [29]. In our studies starvation also significantly increased the expression of the INSr in CA. Our data support a simple model that describes the relationship among starvation, decrease in insulin signaling and decrease in JH synthesis (Fig. 7).

There is evidence that the ISP affects JH levels; in Drosophila, a mutation of the INSr resulted in a significant decrease in JH synthesis, reduced reproductive output and extended life-span [26,27]. Systemic INSr and TOR RNAi decreased JH contents in honey bee [28]. In termites, insulin signaling was increased by JH application, suggesting that there is a feedback loop connecting JH and insulin signaling and that the ISP may also act downstream of JH [29]. Interplays between insulin signaling, JH and environmental cues (including nutrition) were also described in ants [30]. On the other hand, a direct effect of manipulation of the ISP in the CA has been only described in mosquitoes and cockroaches. Systemic TOR knockdown phenocopies starvation and decreases JH synthesis in the cockroach Blatella germanica [31]. In addition, FOXO knockdown in starved cockroaches elicited an increase of JH biosynthesis; implying that FOXO plays an inhibitory role on JH biosynthesis during starvation [32].

In female adult mosquito, the interactions between JH, insulin, nutrition and oogenesis are diverse depending on the tissue and physiological stage. After adult emergence, JH connects the nutritional status with insulin signaling by inducing the expression of TOR signaling components in the fat body. Thus, through JH action, the fat body becomes competent to respond to insulin and transduce the nutritional signal from a blood meal to activate vitellogenin transcription [33]. Later, during vitellogenesis, ILP3 dose-dependently stimulates yolk uptake by oocytes and ecdysteroid production by the mosquito ovaries. ILP3 also exhibits metabolic activity by elevating carbohydrate and lipid storage [10]. In vivo knockdown of the mosquito insulin receptor by RNAi delayed but did not fully inhibit trypsin-like gene expression in the midgut, ecdysteroid production by ovaries and vitellogenin expression by the fat body. In contrast, in vivo treatment with double-stranded INSr RNA and rapamycin completely blocked egg production [34].

Manipulations that decrease systematically the activity of nutrient sensing system are often difficult to interpret. Depletion of INSr or FOXO has profound consequences beyond reducing the activity of any of these particular genes. We observed that silencing of FOXO very efficiently reduced both FOXO and INSr mRNAs in starved and sugar-fed adult females. That explains that although starvation clearly increased FOXO transcript levels in the CA with a concomitant decrease in JH synthesis, systemic silencing of FOXO decreased INSr levels, phenocopied starvation and reduced JH synthesis in both sugar-fed and starved females. Interestingly, systemic silencing of the INSr had opposite effects in JH synthesis by CA dissected from sugar-fed and starved females. Insulin stimulates JH synthesis, so a decrease in the biosynthetic activity by the CA of sugar-fed females deficient in INSr is expected. On the other hand, the significant increase in JH synthesis by the CA of starved females deficient in INSr might be explained by a systemic depletion of INSr in tissues such as brain, fat body or ovaries that might affect organismal nutrient sensing and feed back into CA physiology.

JH synthesis by the CA of female mosquitoes displays remarkable dynamic changes. The CA needs to adjust its synthetic activity to generate these dynamic changes [11,12,35]. A coordinated expression of most JH biosynthetic enzymes has been described in female pupae and adult mosquitoes; increases or decreases in transcript levels for all the enzymes were concurrent with increases or decreases in JH synthesis [35]. The rate of JH biosynthesis is controlled by the rate of flux of isoprenoids in the pathway, which is the outcome of a complex interplay of changes in precursor pools, enzymatic activity levels and external regulators [12,35,36,37,38,39]. The spontaneous rate of JH synthesis is always markedly lower that the maximum inducible rate observed when the CA is stimulated with precursors or activators such as insulin [35,13]. Reducing insulin signaling with PI3K inhibitors rapidly reduced JH biosynthetic enzyme transcripts; validating that a transcriptional regulation of the genes encoding JH biosynthetic enzymes is at least partially responsible for the dynamic changes of JH biosynthesis [13]. Under starvation conditions, the CA showed a reduction of transcript levels for JHAMT, but the synthetic potential of the CA remained unaffected and the gland rapidly and efficiently responded to insulin stimulation by increasing JH synthesis to rates similar to those of CA from non-starved females.

In summary our studies are providing new insights into the regulatory circuitry that controls both JH synthesis and reproductive output in response to nutrient availability. The decrease of JH

![Figure 7. Starvation effects on insulin signaling components and JH synthesis in the CA of mosquitoes.](image)
synthesis in response to starvation is primarily caused by a reduction on insulin signaling and therefore can be completely reversed by stimulation with insulin. JH primarily controls nutrient allocation into the ovaries and starvation reduces JH synthesis and redirects resources away from reproduction. The CA of starved females remains fully competent to synthesize JH; starvation triggers the up regulation of INSr mRNA in CA cells, so they became more sensitive to insulin signaling, providing a mechanism to amplify insulin signals by allowing their detection in lower concentrations once nutrients are acquired and JH synthesis needs to be reactivated.

Supporting Information

Table S1 Primers used for amplification, quantification of the genes studied mRNA and to make dsRNA. (DOCX)

Acknowledgments

The authors thank Dr. Marcela Nouzova for rearing mosquitoes and for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: MPH FGN. Performed the experiments: MPH CRP. Analyzed the data: MPH CRP FGN. Wrote the paper: MPH FGN.

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