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Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*

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Abstract

In mosquitoes, yolk protein precursor (*YPP*) gene expression is activated after a blood meal through the synergistic action of a steroid hormone and the amino acid/Target of Rapamycin (TOR) signaling pathway in the fat body. We investigated the role of insulin signaling in the regulation of *YPP* gene expression. The presence of mosquito insulin receptor (InR) and the Protein kinase B (PKB/Akt) in the adult fat body of female mosquitoes was confirmed by means of the RNA interference (RNAi). Fat bodies stimulated with insulin were able to promote the phosphorylation of ribosomal S6 Kinase, a key protein of the TOR signaling pathway. Importantly, insulin in combination with 20-hydroxyecdysone activated transcription of the *YPP* gene vitellogenin (*Vg*), and this process was sensitive to the Phosphoinositide-3 kinase (PI-3k) inhibitor LY294002 as well as the TOR inhibitor rapamycin. RNAi-mediated knockdown of the mosquito InR, Akt, and TOR inhibited insulin-induced *Vg* gene expression as well as S6 Kinase phosphorylation in *in vitro* fat body culture assays.

Keywords

insulin receptor; Akt; S6K; target of rapamycin; RNAi

1. Introduction

Diseases caused by mosquitoes have a profound influence on human health worldwide. Repeated cycles of blood feeding and egg development make mosquitoes an efficient vector by which pathogens can spread from one host to another. Therefore, elucidation of the physiological and molecular mechanisms underlying the blood meal regulation of events and genes essential for vitellogenesis and egg maturation is of paramount importance in our efforts to develop novel approaches to vector and pathogen control.

Females of the anautogenous yellow fever mosquito, *Aedes aegypti*, require blood in order to develop eggs and, hence, reproduce. The central step in mosquito reproduction is vitellogenesis. It involves the synthesis and secretion of yolk protein precursors (YPP) on a massive scale by the fat body, a tissue analogous to the vertebrate liver (Raikhel et al., 2002). This event is followed by the accumulation of yolk in the developing oocytes (Raikhel & Dhadialla, 1992).

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YPP gene transcription is regulated by hormonal as well as nutritional cues. After a blood meal, the stretching of the midgut walls results in a so-far unknown blood-meal-dependent signal that stimulates neural tissues in the brain to release a neuropeptide hormone known as ovarian ecdysteroidogenic hormone (OEH) into the hemolymph (Brown et al., 1998). OEH stimulates the ovaries to produce the insect steroid hormone ecdysone. Ecdysone is converted into its active form 20-hydroxyecdysone (20E) in the fat body. At the same time, amino acids from the blood meal directly signal to the fat body, which in conjunction with 20E stimulates the transcription of *YPP* genes (Attardo et al., 2005).

Transcript levels of the vitellogenin (*Vg*) gene, a major *YPP*, increase greatly after a blood meal, reach their peak at around 24 h post-blood meal (PBM), and rapidly decline thereafter (Cho & Raikhel, 1992). *Vg* transcript expression follows the changing titers of the steroid hormone 20E (Li et al., 2000). 20E works directly through its heterodimeric nuclear receptors, ecdysone receptor protein (EcR) and ultraspiracle (USP) (Wang et al., 1998; Wang et al., 2000). Analysis of the *Vg* gene promoter region reveals the presence of binding sites for EcR complex (EcR/USP), the products of 20E-stimulated early genes, E74 and E75, as well as GATA-type transcription factors (Kokoza et al., 2001; Martin et al., 2001).

Nutrition in the form of a blood meal plays a very important role in mosquito egg development. The insect “fat body” is known to be the nutrient sensor organ (Edgar, 2006). Nutritional signal, inside the cell cytoplasm can be conveyed by two main signaling pathways: the amino acid signaling pathway and the insulin signaling pathway. Previous work has shown that amino acid signals are transduced in the mosquito fat body cells through the Target of Rapamycin (TOR) protein (Hansen et al., 2004). Inhibiting TOR either by the drug rapamycin or by RNAi-mediated knockdown resulted in a severe down-regulation of *Vg* gene expression after amino acid stimulation in an *in vitro* fat body culture system. TOR depletion also resulted in smaller ovaries as well as a reduced number of deposited eggs after a blood meal (Hansen et al., 2004). One of the major downstream target molecules of TOR is the ribosomal protein S6 kinase (S6K) which phosphorylates the ribosomal protein S6 (Hansen et al., 2005; Volarevic & Thomas, 2001; Zhang et al., 2000). There is direct correlation between the amino acid signaling and S6K phosphorylation through TOR after a blood meal in the fat body and ovaries of *Aedes aegypti*. RNAi-mediated *AaS6K* down-regulation effectively blocks mosquito egg development after a blood meal (Attardo et al., 2005; Hansen et al., 2005).

The insulin signaling pathway is conserved in eukaryotic organisms from yeast to mammals (Garofalo, 2002). In *Drosophila melanogaster*, the insulin signaling pathway plays a major role in regulating growth, development, metamorphosis, female fertility, and longevity (Bohni et al., 1999; Burks et al., 2000; Chen et al., 1996; Clancy et al., 2001; Tatar et al., 2001). Recent studies have identified seven insulin-like peptides (DILPs) in *Drosophila* (Garofalo, 2002). In insects, the neurosecretory cells in the brain are believed to be the major source of ILPs, as reported in various immuno-cytochemical studies. DILPs are peptides that resemble human insulin rather than IGF1 or IGF2, which are single polypeptides (Brogiolo et al., 2001). Of the seven DILPs, DILP2 is the most closely related, with 35% identity to mature insulin. Nucleotide sequences encoding ILPs have been identified from both the *Anopheles gambiae* and the *Aedes aegypti* genome databases (Riehle et al., 2002; Riehle et al., 2006). Of the eight genes that encode for the *Aedes* ILPs, seven have the pro-peptide structure consistent with the other invertebrate and vertebrate ILPs (Riehle et al., 2006).

Homologues of vertebrate insulin receptors have been cloned and characterized from *Drosophila*, *Caenorhabditis elegans* as well as from the mosquito *Aedes aegypti* (Gregoire et al., 1998; Nishida et al., 1986; Graf et al., 1997). The mosquito InR in *Aedes* is a protein of approximately 400 kDa consisting of two α and two β subunits (Riehle & Brown, 2002). The α -subunit has a conserved ligand-binding domain while the β -subunit houses a tyrosine kinase

domain. Protein and transcripts of InR have been found primarily in the ovaries, but its transcripts have also been observed in the head and body wall of females (Graf et al., 1997). A key component of the insulin signaling pathway, the protein kinase B (PKB), commonly known as Akt, was identified and cloned from the ovaries of *Aedes aegypti* (Riehle and Brown 2003). In addition, a functional Phosphoinositide-3 kinase (PI-3k) in the mosquito fat body has also been identified (Hansen et al., 2005).

Until now, little has been known about the functional role of insulin pathway in mosquitoes. In this paper, we report that insulin induces the phosphorylation of S6K, a key downstream target molecule of TOR in the fat body of *Ae. aegypti*. Surprisingly, insulin in combination with 20E significantly enhances *Vg* gene transcription in *in vitro* fat body culture, and pharmacological inhibitors of insulin signaling inhibit these effects. Moreover, we show that RNAi-mediated InR and *Akt* gene knockdowns effectively block stimulation of *Vg* gene expression by insulin and 20E.

2. Materials and methods

2.1. Mosquito rearing and in vitro fat body culture

The *Ae. aegypti* mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described by Hays and Raikhel (1990). The mosquitoes were reared at 27 °C and 85% relative humidity. Adults were fed on 10% sucrose through a wick. The *in vitro* cultures were performed using a total of nine mosquitoes (three groups of three mosquitoes) for each treatment. Dissection was performed in *Aedes* physiological saline at room temperature (see below). The three fat bodies in a group were kept in a single well in a tissue culture plate and were incubated in a total of 100 µl of the appropriate medium (see below) at 27 °C for 3 h. All the experiments were repeated at least three times with three unique cohorts of mosquitoes.

2.2. Reagents used for in vitro fat body culture

The following reagents were used: bovine insulin solution (Sigma, St. Louis, MO), Corazonin (Sigma), TOR-inhibitor rapamycin (Cell Signaling, Danvers, MA), PI-3k inhibitor LY294002 (Calbiochem, San Diego, CA), and 20-hydroxyecdysterone (concentration used for all experiments, 1 µM; Sigma). For inhibitor studies, the dissected fat bodies were incubated in *Aedes* physiological saline (APS) with the appropriate inhibitor drug for 1 h. After this time, the fat bodies were washed with APS and incubated in medium lacking amino acids (containing equimolar amounts of mannitol in place of amino acids) (Attardo et al., 2005) together with the appropriate inhibitor or other reagents. From hereon, this medium will be referred to as amino acid negative media (AA⁻), while that containing amino acids will be referred to as amino acid plus medium (AA⁺).

Composition for *Aedes aegypti* incubation media:

- A. *Aedes* physiological saline (APS): 150mM NaCl, 4mM KCl, 0.1mM NaHCO₃, 0.6mM MgCl₂, 25mM HEPES buffer, pH adjusted to 7.0 at 27 °C with NaOH; CaCl₂ added to 1.7mM
- B. Fat body culture media (For making AA⁺ and AA⁻ media): i) Salt stock solution – NaCl (137mM), KCl (1mM), MgCl₂.6H₂O (0.6mM) and NaHCO₃ (1.8mM); ii) Calcium Stock solution- CaCl₂.2H₂O (1.2 mM); iii) TES buffer stock solution- K₂HPO₄ (2mM) and TES (25mM). Solutions were filtered through bacterial filter (0.22µm pore size) and stored at 4 °C.

2.3. RNA extraction, reverse transcription and real-time PCR

Total RNA from dissected mosquito fat bodies was extracted after homogenization with a motor-driven pellet pestle mixer. For quantitative PCR, three groups of three fat bodies were used for each treatment. All quantitative PCR experiments were replicated with at least three unique cohorts of mosquitoes. Total RNA was isolated by means of a commercially available modification (TRIzol, Invitrogen, Carlsbad, CA) of the one-step phenol/guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). Aliquots of 1 µg total RNA treated with amplification-grade RNase-free DNase I (Invitrogen) were used in the cDNA synthesis reactions using an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). Reverse transcription was carried out according to the manufacturer's protocol in 20-µl reaction mixtures containing random primer or oligo (dT) primer at 37°C for 1 h. PCR products were obtained from the PCR machine using a HotStar Taq Master Mix kit (Qiagen), and 1 µl of cDNA was subjected to PCR using specific primers. PCR products were separated on 1% agarose gels.

Real-time PCR was performed using the iCycler iQ system (Bio-Rad, Hercules, CA), and reactions were performed in 96-well plates using TaqMan primers/probes for *Vg*, and SYBR green primers for *S7* ribosomal protein (internal control). We used a real-time PCR master mix, iQ Supermix (Bio-Rad) for the TaqMan reactions or the iQ SYBR green supermix (Bio-Rad) for the SYBR green reactions. Quantitative measurements were performed in triplicate and normalized to the internal control of *S7* ribosomal protein mRNA for each sample. Primers and probes were as follows (all TaqMan probes used the Black Hole Quencher and were synthesized by Qiagen: *Vg* forward, 5'-ATGCACCGTCTGCCATC; *Vg* reverse, 5'-GTTCTAGTTGGAAAGCTCG; Texas Red labeled *Vg* probe, 5'-AAGCCCCGCAACCGTCCGTACT; *S7* forward, 5'-TCAGTGTACAAGAAGCTGACCGGA; *S7* reverse, 5'-TTCCGCGCGCTCACTTATTAGATT. Reactions were carried out as described previously (Attardo et al., 2003). Real-time data were collected by the ICYCLER IQ REAL TIME DETECTION SYSTEM SOFTWARE V3.0 for WINDOWS. Raw data were exported to EXCEL (Microsoft) for analysis.

2.4. Protein extraction and Western blot analysis

Total protein extracts were prepared from dissected fat bodies. Fat bodies were isolated and subjected to different treatments in fat body culture. At the end of the culture period, groups of nine fat bodies were homogenized in 100 µl of breaking buffer (50 mM Tris pH 7.4; 1% IGEPAL; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonylfluoride; 1X protease inhibitor mixture; 1X phosphatase inhibitor mixture, Sigma) using a pellet pestle. The homogenate was boiled in 25 µl LDS (4X) NuPage sample buffer (Invitrogen) with 12.5 µl (10X) sample reducing agent (Invitrogen) for 5–10 min. Insoluble material was removed using QiaShredder columns (Qiagen). Supernatant was removed, and protein concentration was determined by means of a Bradford assay (Bio-Rad). Aliquots of the protein extract were resolved on 4–15% gradient SDS-polyacrylamide gels (Bio-Rad) and electrotransferred to PVDF membranes. The membranes were blocked overnight in Starting Block™ T20 (PBS) blocking buffer (Pierce, Rockford, IL) at 4°C. After washing with PBS/T, the membranes were incubated with the primary antibody in the Starting Block™ T20 overnight at 4°C. The bands were visualized using ECL reagents (Pierce).

Western blot analysis was performed using an antibody that detects phosphorylated S6 kinase (Thr-412; number 07–018, Upstate, Lake Placid, NY). This polyclonal antibody detects the human p70 S6 kinase phosphorylated on Thr 412 and the human splice variant of human p70 S6 kinase phosphorylated on Thr 389 [Thr 388 in *Aedes*, (Hansen et al., 2005)]. An antibody

against native S6 kinase protein was also used for loading controls (p70 S6K antibody from Cell Signaling, catalog no. 9202).

2.5. RNAi knockdown experiments

For production of dsRNA, PCR primers were synthesized against specific regions of *AaInR*, *AaAkt*, and *AaTOR*, with T7 minimal primers attached to both the 5' and 3' ends. Primers were chosen such that the PCR products would be between 500 and 600 nucleotides in size. The PCR products were then cleaned and concentrated using the Qiagen PCR cleanup and gel extraction kits. Finally, double strands of RNA were produced by means of *in vitro* transcription with the T7 RNA polymerase using the MEGAscript T7 kit (Ambion, Austin, TX, USA). dsRNA from a small portion of the bacterial gene *Mal* (*dsMal*) was used as a negative control. The control dsRNA *Mal* was amplified from the plasmid 28iMal (NEB). It contains a non-functional part of the *E. coli MalE* gene that encodes the maltose-binding protein. Approximately, 1 µg dsRNA in 0.3–0.5 µl of distilled water was injected into the thorax of CO₂-anesthetized female mosquitoes 1 or 2 days after emergence. The dsRNA-injected mosquitoes were allowed to recover for 5 days and then were dissected for fat body culture.

3. Results

3.1. Insulin induces phosphorylation of S6K in cultured fat bodies

In the mosquito fat body, ribosomal S6K is phosphorylated after amino acid-stimulation in a TOR-dependent manner (Hansen et al., 2005). We examined the effect of bovine insulin on S6K phosphorylation. Previously, several studies have reported the use of mammalian insulin in different insect species. Bovine and porcine insulin have been successfully used for characterizing the insulin signaling pathway in *Drosophila* and *Ae.aegypti* (Graf et al., 1997; Radimerski et al., 2002).

Fat bodies of female mosquitoes, 72 h after eclosion, were isolated, and the samples were kept in APS for 1 h at 27°C. After this, APS was replaced by either AA+ or bovine insulin solution. Fat bodies incubated only with AA– served as the negative control. After 3 h of incubation, the fat bodies were homogenized in protein breaking buffer and subjected to western blot analysis using a phospho-S6K antibody. The full length open reading frame of *Aedes* S6K protein consists of 550 amino acids and its molecular weight is predicted to be around ~62 kDa (Hansen et al., 2005). We found significant amounts of phospho-S6K after stimulation with insulin compared with that in the negative control (Fig. 1A).

Next, we investigated whether the PI-3 kinase inhibitor LY294002 could block insulin-induced S6K phosphorylation. Cultured fat bodies were pre-treated in APS containing LY294002; 100, 200, 500 nM, 1, 2, 5 µM (Fig. 1B) and 5, 10, 20, 50, 100 µM (data not shown) for 1 h and then stimulated with medium containing insulin solution and varying concentrations of LY294002 (see above) for 3 h. We found that LY294002 acts as a strong inhibitor of insulin-induced S6K phosphorylation at a concentration of 5 µM and above (Fig. 1B).

In order to verify the specificity of insulin's action further, we incubated fat bodies with insulin solution (17 and 100 µM) and heat-inactivated insulin. Bovine insulin solution (100µM) was boiled for 10 minutes, cooled to room temperature and this heat-inactivated insulin solution was then used in the culture media instead of the original peptide hormone. Fat bodies were cultured in a manner similar to that described above in AA– medium and total protein was extracted. S6K phosphorylation was analyzed through western blotting. Interestingly, we did observe an increase in S6K phosphorylation with 100 µM insulin compared with that seen with 17 µM. However, fat bodies incubated with heat-inactivated insulin solution showed no S6K phosphorylation (Fig. 1C). Furthermore, we also wanted to determine whether this

phosphorylation effect could be initiated by any other peptide hormone other than insulin. An unrelated peptide hormone corazonin was used in this respect. We chose this peptide hormone as it is secreted from the brain neurosecretory cells, signals through its own specific receptor, circulates in the hemolymph and is widely found in many insect species including *Drosophila melanogaster* (Hua et al., 2000). This hormone was first identified as a cardioaccelerator in common cockroaches and has been shown to stimulate the release of pre-ecdysis and ecdysis triggering hormones within the Inka cells of *Manduca sexta* (Kim et al., 2004). Dissected fat bodies were incubated with corazonin (17 μ M and 100 μ M) for 3 h in *in vitro* fat body culture system and total protein extracted. Similarly as before, western blot analysis was performed to determine the level of S6K phosphorylation. No phosphorylation of S6K was observed with up to 100 μ M of corazonin (Fig. 1C).

3.2. Insulin activates Vg gene expression in mosquito fat bodies in vitro only together with 20E

Next, we investigated whether insulin was capable of stimulating *YPP* gene expression. Fat bodies from 3- to 5-day-old female mosquitoes were incubated in AA⁻ medium with different combinations of insulin and/or 20E for 3 h at 27°C. Total RNA was collected and subjected to real-time PCR analysis to determine the level of *Vg* gene expression. Our results show that neither insulin nor 20E alone had any effect on stimulating *Vg* expression. Only when insulin was given together with 20E did we notice a significant increase in *Vg* mRNA level (Fig. 2A).

To confirm the specificity of insulin's action, we treated fat bodies with the PI-3 kinase inhibitor LY294002. To determine the optimal concentration of this drug, we performed a titration experiment. Dissected fat bodies were pre-incubated with different concentrations of LY294002 in APS for 1 h and then stimulated with insulin and 20E in the presence of the drug for 3 h in AA⁻ media. We found that 5 μ M LY294002 was sufficient to inhibit the stimulation of *Vg* gene transcription caused by insulin and 20E (Fig. 2B). To test whether the fat body tissue remained alive in the presence of 5 μ M LY294002, we also confirmed its ability to phosphorylate S6K in the presence of amino acids (not shown).

3.3. RNAi-mediated knockdown of AaInR and AaAkt inhibits vitellogenin gene activation by insulin and 20E

To test whether bovine insulin signals through the mosquito insulin receptor (InR), we utilized the RNAi-mediated knockdown technique. Female mosquitoes, 1–2 days after eclosion were injected with either dsRNA against InR or a control dsRNA (*Mal*). The control dsRNA (*Mal*) was used to rule out any possible non-specific effect caused by RNAi injections. After a 5-day recovery period, fat bodies were isolated and kept in APS for 1 h at 27°C. The APS was replaced by AA⁻ media lacking or containing insulin and 20E. After a 3-h incubation period, RNA was isolated from the fat bodies. Relative levels of *Vg* mRNA were determined via quantitative PCR. Fat bodies of InR-knockdown mosquitoes showed significant reduction in *Vg* gene expression when compared with the *Mal* control and when treated with media containing insulin and 20E (Fig. 3A). Incubation with medium containing insulin and 20E resulted in a strong up-regulation of *Vg* mRNA in the *Mal*-injected mosquitoes. This up-regulation was completely blocked in the InR RNAi-treated fat bodies.

To further investigate the role of components of the insulin signaling pathway in *Vg* gene regulation, we used the RNAi approach to knockdown *AaAkt* in unfed female mosquitoes. The *AaAkt* transcript was found to be fairly abundant in the mosquito fat body (data not shown). Isolated fat bodies from RNAi-treated mosquitoes were subjected to *in vitro* fat body culture and incubated in AA⁻ medium in the presence or absence of insulin, 20E, or both. *AaAkt* knockdown resulted in a strong reduction of *Vg* gene expression after insulin and 20E stimulation when compared with mosquitoes treated with control dsRNA (Fig. 3B). S₇

ribosomal protein was used as the internal control for the real-time PCR analysis. Although the transcript level of *S7* ribosomal protein has been shown to fluctuate only after a blood meal (unpublished data), its levels did not vary significantly in our *in vitro* studies, even in the presence of hormonal and inhibitor treatments (data not shown).

3.4. Insulin signals via the TOR kinase

To examine whether the TOR kinase is a part of the signaling pathway that transduces the insulin signal to the *Vg* gene in the mosquito fat body in the absence of amino acid signaling, we treated cultured fat bodies with rapamycin (150 nM) in the presence of insulin and 20E in AA– medium. Total RNA was collected from the cultured fat bodies and was subjected to quantitative real-time PCR. Rapamycin treatment resulted in a significant reduction of the *Vg* gene stimulation brought about by insulin and 20E when compared with the control (Fig. 4A).

The effect of the TOR-inhibitor rapamycin on insulin-induced S6K phosphorylation was investigated by treating isolated fat bodies with 150 nM rapamycin. After a pre-treatment in APS lacking or containing rapamycin for 1 h, the fat bodies were stimulated with AA– medium containing either insulin (17 and 100 μ M) or insulin with rapamycin for 3 h. Addition of 150 nM rapamycin resulted in the suppression of insulin-induced increased phosphorylation of S6K. Rapamycin completely inhibited *AaS6K* phosphorylation even in the presence of higher concentrations of insulin (Fig. 4B).

To further confirm the role of the TOR kinase in fat body insulin signaling, we used the RNAi method to knockdown *AaTOR* mRNA. dsRNA against *AaTOR* was injected into the thorax of anesthetized female mosquitoes (1–2 days old). After a 5-day recovery period, fat bodies were dissected and cultured in medium containing insulin and 20E. Mosquitoes injected with *dsInR* and fat bodies treated with the drug rapamycin were used as additional controls for this experiment. Total RNA was collected as described above and analyzed for *Vg* transcript expression using real-time PCR. *AaTOR* knockdown resulted in complete inhibition of the *Vg* gene expression relative to that of the control (*Mal*) (Fig. 5A).

LY294002 and rapamycin inhibit S6K phosphorylation when stimulated by insulin solution (see above). We utilized the RNAi approach to test the role of *AaInR*, *AaAkt*, and *AaTOR* in S6K phosphorylation. RNAi-mediated down-regulation of all three genes completely inhibited S6K phosphorylation after stimulation by insulin in fat bodies incubated in the AA– media in the presence of insulin (Fig. 5B). Fat bodies injected with *dsMal* and from non-injected mosquitoes (wild type-WT) were used as control.

4. Discussion

In this study, we have identified insulin signaling as a component involved in the regulation of *YPP* gene expression in the fat body of female mosquitoes. We used an *in vitro* fat body culture system to test the effects of insulin directly on fat body tissue. Treatment with a combination of insulin and 20E resulted in a strong up-regulation of *Vg* gene expression in the fat body, even in the absence of amino acids. This up-regulation was not observed when 20E or insulin was given alone and was sensitive to the PI3-K-inhibitor LY294002.

In our earlier work, we found that amino acid stimulation of fat bodies *in vitro* results in a strong increase in phospho-S6K and that this rise is sensitive to the TOR-inhibitor rapamycin (Hansen et al., 2005). Here, we have demonstrated that stimulation with insulin triggers S6K-phosphorylation *in vitro* in the absence of amino acids. Mammalian cells require nanomolar quantities of bovine insulin to initiate any type of cellular or physiological responses. Keeping in mind that bovine insulin is a distantly heterologous ligand of the *InR*, it is not surprising to

observe that the optimum concentration required to stimulate physiological responses in *Aedes aegypti* fat body cells is in micromolar amounts. In *Aedes*, bovine insulin was shown to stimulate ecdysteroid production from the ovaries in a dose-dependent manner and about 17 μ M of bovine insulin constituted the optimum concentration to induce this effect (Riehle & Brown, 1999).

The next question we addressed was whether the insulin effects we observed in these experiments were due to the activation of the mosquito *InR* and the serine/threonine kinase - Akt. The *InR* and Akt are expressed in the follicle cells of the ovaries of *Ae. aegypti* and to a lesser degree in other tissues (Graf et al., 1997; Riehle & Brown, 2003). We show that the *InR* is present and active in the mosquito fat body and can promote *YPP* gene transcription. RNAi-mediated depletion of both *InR* and Akt in the fat body resulted in strongly reduced *Vg* gene transcription after stimulation with insulin and 20E; treatment with rapamycin and RNAi-mediated knockdown of TOR had a similar effect. These results suggest that insulin acts through the mosquito *InR*, which transduces the signal via Akt, TOR, and S6K. In this respect, our research confirms the results of other studies showing that the insulin/*InR* signaling pathway is highly conserved between mammals and insects (Shingleton, 2005; Stocker & Hafen, 2000; Wu & Brown, 2006). Although it is clear that insulin acts via a conserved pathway, the mechanism of the synergistic action of insulin and 20E remains to be elucidated. However, our study has revealed a novel and unexpected regulatory link between the insulin signaling and the activation of the *Vg* gene transcription in the mosquito fat body.

Our data have shown that the insulin signaling in the mosquito fat body requires the TOR kinase as an indispensable signal transducer for downstream signaling (Hansen et al., 2004). The serine/threonine protein kinase TOR is one of the best characterized and arguably the most important regulatory target of insulin signaling (Oldham et al., 2000; Oldham & Hafen, 2003). Amino acids have been known to positively regulate TOR either directly or through other intermediate signaling molecules (Arsham & Neufeld, 2006). Through our pharmacological studies with the drug rapamycin (TOR-inhibitor), we have shown that there was no phosphorylation of S6K in the presence of insulin. *Vg* gene transcription was also reduced by a significant amount in the presence of the drug and insulin together with 20E. We confirmed our previous observations through RNAi-mediated knockdown studies. TOR knockdown completely inhibited both S6K phosphorylation and *Vg* gene transcription *in vitro*. These results suggest that insulin signaling converges with the amino acid signaling at the level of TOR kinase. At this point, it is imperative to mention that previous findings in *Drosophila* and in mammals have shown that the Tuberous Sclerosis Complex 2 (TSC2) is a key target of regulation by many of the upstream inputs shown to regulate TOR and/or S6K activity (Dan et al., 2002; Inoki et al., 2002; Potter et al., 2002). TSC2 is phosphorylated on several sites by Akt after insulin stimulation (Avruch et al., 2006). It has previously been shown that down regulation of TSC2 in mosquitoes had an opposite effect on the *Vg* gene expression than did that of TOR. Activation of the *Vg* gene in TSC2-knockdown mosquitoes was significantly stronger in response to stimulation by amino acids in the *in vitro* fat body culture system (Hansen et al., 2004). Hence, it is possible that the insulin signaling actually merges with the amino acid signaling pathway through the TSC complex rather than directly influencing TOR kinase itself. Further experiments on these intermediate signaling molecules connecting TOR and Akt would possibly reveal the entire signaling mechanism in the fat body.

In conclusion, the results of our present work suggest that insulin signaling may act in combination with amino acids and 20E as a third component regulating *YPP* gene expression in female mosquitoes.

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Fig. 1. Insulin stimulates AaS6K-phosphorylation in *in vitro* fat body culture

(A) Fat bodies from 3- to 5-day-old females were dissected and incubated in the presence or absence of insulin. A total of nine mosquito fat bodies were subjected to experimental treatment for each unique cohort of mosquitoes. All the western blot experiments were performed using at least three unique cohorts of mosquitoes at different times. Fat bodies incubated only with complete medium, i.e., medium with amino acids (AAs), were treated as a positive control. Western blot analyses were performed using phospho-S6K antibodies (upper panels). A second blot was probed with an antibody against the S6K protein as loading controls (lower panels). Concentration of insulin used: 17 µM. (B) **The PI-3k inhibitor LY294002 can efficiently inhibit S6K-phosphorylation at a minimum concentration of 5 µM.** Western blot analyses

continued as described above; dissected fat bodies were incubated with increasing concentration of the PI-3k inhibitor drug LY294002. (C) **Neither heat-inactivated insulin nor corazonin can induce S6K phosphorylation in *in vitro* cultured fat bodies.** *AaS6K* is specifically phosphorylated in the presence of the active hormone: insulin solution. Western blot analyses were performed as described above. Dissected fat bodies were incubated separately in 17 and 100 μ M insulin, 17 and 100 μ M corazonin (unrelated peptide hormone) and 100 μ M heat-inactivated insulin.

Fig. 2. The mosquito fat body responds to insulin by stimulating *Vg* gene expression *in vitro*
 (A) *Vg* gene responsiveness to insulin depends on the presence of 20E. Real-time PCR analysis of *Vg* mRNA from *in vitro* cultured mosquito fat bodies in the presence or absence of insulin (17 μ M) and/or 20E (1 μ M). Fat bodies from 3- to 5-day-old mosquitoes were pre-incubated for 1 h in untreated physiological saline (APS). After 1 h, the fat bodies were washed with untreated saline and incubated with appropriate culture media containing insulin and/or 20E for 3 h. Total RNA was collected and cDNA was synthesized from equal amounts of DNase-I-treated total RNA. Data were normalized by means of real time PCR analysis of ribosomal *S7* gene levels in the cDNA samples. Data represent means $\pm$ SE of triplicate samples from three unique cohorts of mosquitoes. (B) Insulin responsiveness is dependent on the presence

of 20E and can be inhibited by the PI-3 kinase antagonist LY294002. A titration experiment was performed with this drug to determine the minimal optimum concentration required to augment this inhibition. Real-time PCR analysis of *Vg* mRNA from *in vitro* cultured mosquito fat bodies was performed with or without LY294002 in the presence or absence of insulin (17 μ M) and 20E (1 μ M). Fat bodies from 3- to 5-day-old mosquitoes were pre-incubated for 1 h in either untreated physiological saline (APS) or saline containing the appropriate concentration of LY294002. After 1 h, the fat bodies were washed with untreated saline and incubated with appropriate culture media containing insulin, 20E, and/or LY294002 for 3h. RNA isolation and real-time PCR analysis were performed as in (A).

Fig. 3. RNAi-mediated knockdown of *AaInR* and *AaAkt* inhibits *Vg* mRNA expression *in vitro*
 (A) Depletion of *AaInR* inhibits *Vg* gene expression *in vitro*. Female mosquitoes were injected with 1 μ g dsRNA duplexes of either ds*AaInR* or a control dsRNA derived from the non-coding region of a bacterial gene (ds*Mal*) and incubated for 5 days. Fat bodies were dissected and subjected to *in vitro* fat body culture with medium containing or lacking insulin (17 μ M) and 20E (1 μ M) for 3 h. *Vg* mRNA levels were determined by means of real-time PCR. Data represent $\pm$ SE of triplicate samples from three unique cohorts of mosquitoes. Depletion of *AaInR* severely reduces *Vg* gene transcription *in vitro* upon stimulation by insulin and 20E. RT-PCR analysis shows *AaInR* expression in fat bodies injected with control *Mal* dsRNA and *AaInR* dsRNA; the *S7* gene is used as an internal control. (B) RNAi-mediated knockdown of

AaAkt significantly decreases *Vg* gene expression. As above, fat bodies of RNAi-treated mosquitoes were stimulated with insulin solution (17 μ M) and 20E (1 μ M) in *in vitro* fat body culture for 3 h. Fat body culture and gene expression analysis was performed as in (A). RT-PCR analysis shows *AaAkt* expression in fat bodies injected with control *Mal* dsRNA and *AaAkt* dsRNA; the actin gene is used as the internal control.

Fig. 4. Rapamycin inhibits insulin-mediated stimulation of *Vg* gene expression and S6K-phosphorylation in *in vitro* fat body culture

(A) The TOR-inhibitor drug rapamycin inhibits *Vg* gene expression in vitro. Fat bodies were isolated and incubated in appropriate culture media for 3 h at 27°C. Real-time PCR analyses were performed to quantify the amount of *Vg* mRNA synthesis in the presence and absence of the drug rapamycin. Statistical studies were performed as illustrated previously. (B) S6K phosphorylation is inhibited in the presence of rapamycin. Western blot analysis was performed using phospho-S6K antibodies (upper panel). A second blot was probed with an antibody against native S6K as loading controls (lower panel). 150 nM rapamycin added to fat body cultures treated with either 17 μM or 100 μM insulin solution. The experiment was replicated three times with unique cohorts of mosquitoes.

Fig. 5. Insulin signals through the TOR signaling pathway in the mosquito fat bodies

(A) RNAi-mediated *AaTOR* knockdown *inhibits* *Vg* gene expression in *in vitro* cultured fat bodies. Female mosquitoes were injected with 1 μ g dsRNA duplexes of either ds*AaTOR* or ds*Mal* and incubated for 5 days. Fat bodies injected with ds*InR* were also used in this experiment for a comparative study. Fat bodies were dissected and subjected to *in vitro* fat body culture with different combinations of media containing or lacking insulin (17 μ M), 20E (1 μ M), and/or rapamycin (150 nM) for 3 h. *Vg* mRNA levels were determined by means of real-time PCR. This analysis shows *AaInR* and *AaTOR* expression in fat bodies injected with control *Mal* dsRNA, *AaInR* dsRNA, and *AaTOR* dsRNA; the *S7* gene was used as an internal control. The quantitative PCR studies for each group were replicated four times with unique cohort of mosquitoes. (B) Depletion of *AaInR*, *AaAkt*, and *AaTOR* inhibits S6K

phosphorylation *in vitro*. Western blot analyses were performed similarly on dsRNA-injected mosquitoes (against *AaInR*, *AaAkt*, and *AaTOR*) to determine the phosphorylation levels of S6K. Female mosquitoes injected with ds*Mal* were used as the control (negative control: without insulin; positive control: with insulin). Wild type (WT) mosquitoes were also kept as an additional positive control as they had not been given any dsRNA injections. Immunoblotting was performed as described above.