The insulin/TOR signal transduction pathway is involved in the nutritional regulation of juvenile hormone synthesis in Aedes aegypti

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1. Introduction

The correct allocation of nutrients between competing needs such as reproduction, growth, maturation or flight is a vital component of an insect's life-history strategy (Boggs, 2009; Clifton and Noriega, 2011). Juvenile hormone (JH) has been described as part of a transduction system that assesses nutritional information and regulates reproduction in mosquitoes (Noriega, 2004). JH synthesis and ovarian previtellogenic maturation are activated in newly eclosed Aedes aegypti adult females only if teneral nutritional reserves are elevated (Caroci et al., 2004). Later, after previtellogenic maturation has been completed, JH mediates reproductive trade-offs in resting stage mosquitoes in response to nutritional information from mosquitoes (Clifton and Noriega, 2012). JH titers in female adult mosquitoes are mainly determined by the rate at which the corpora allata (CA) synthesize JH (Li et al., 2003a). Adult female A. aegypti show dynamic changes in JH biosynthesis (Li et al., 2003a). 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; suggesting that transcriptional changes are at least partially responsible for the dynamic changes of JH biosynthesis. The goal of the present study is to identify signaling network components responsible for the nutritional-dependent changes of JH synthesis in the CA of mosquitoes. The insulin/TOR signaling network plays a central role in the transduction of nutritional signals that regulate cell growth and metabolism in insects. These pathways have also been suggested as a link between nutritional signals and JH synthesis regulation in the CA of cockroaches and flies. We used a combination of in vitro studies and in vivo genetic knockdown experiments to explore nutritional signaling pathways in the CA. Our results suggest that the insulin/TOR pathway plays a role in the transduction of the nutritional information that regulates JH synthesis in mosquitoes. Transcriptional regulation of the genes encoding JH biosynthetic enzymes is at least partially responsible for these nutritionally modulated changes of JH biosynthesis.

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network involving phosphatidylinositol 3-kinase (Riehle and Brown, 1999). Selective activators and inhibitors of insulin signaling cascades had strong effects on insulin-regulated physiological processes in mosquitoes (Riehle and Brown, 1999); for example, knockdown of the A. aegypti phosphatase and tensin homolog (AaegPTEN) affects insulin signaling (Arik et al., 2009).

The insulin/TOR pathway has also been suggested as a link between nutritional signals and JH synthesis regulation in the CA of Blattella germanica (Maestro et al., 2009); as well as a link between diapause signals and JH synthesis regulation in Culex pipiens (Sim and Denlinger, 2008). We used a combination of in vitro studies and in vivo genetic knockdown experiments to explore nutritional signaling pathways in the CA of mosquitoes. Our results suggest that the insulin/TOR pathway plays a role in the transduction of the nutritional information that regulates JH synthesis in mosquitoes. A transcriptional regulation of the genes encoding JH biosynthetic enzymes is at least partially responsible for the insulin/TOR-dependent changes in JH biosynthesis.

2. Material and methods

2.1. Chemicals

Rapamycin was purchased from Sigma–Aldrich (St Louis, MO), bovine insulin from Akron Biotech (Boca Raton, FL) and LY294002 from Calbiochem (Billerica, MA).

2.2. Insects

A. 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 adults were offered a cotton pad soaked in 3% sucrose solution.

2.3. Corpora allata JH bioassays

Corpora allata–corpora cardiaca complexes (CA–CC) attached to the head capsule were isolated from adult females as previously described (Li et al., 2003a). The effects of rapamycin (a TOR inhibitor, 500 nM, dissolved in DMSO), bovine insulin (17 μM, dissolved in HCl) and LY294002 (an inhibitor of insulin signaling, 10 μM dissolved in M-199 medium) were tested at 32 °C in M-199 medium (Lavallette, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and methionine (50 μM). Optimal concentrations were selected by testing 3 different concentrations for insulin (1.7, 17 and 170 μM), rapamycin (150, 500 and 1500 nM) and LY294002 (1, 10 and 100 μM).

2.4. Quantification of JH synthesis

The amount of JH synthesized by CA–CC complexes in vitro was quantified by a recently developed method using high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) (Rivera-Perez et al., 2012). The assay is based on the derivatization of JH III with a fluorescent tag with subsequent analysis by reverse phase HPLC-FD.

2.5. Quantitative PCR (qPCR)

Total RNA was isolated from tissues using RNA-binding glass powder as previously described (Noriega and Wells, 1993). Contaminating genomic DNA was removed using Turbo DNA-free DNase (Ambion, Austin, TX, USA). Reverse transcription was carried out using an oligo dt priming method and SuperScrit+ III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA). qPCR was performed with a 7300 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. Standard curves to quantify relative gene copy number were made from serial dilutions of plasmids containing the mosquito genes (300,000; 30,000; 3000; 30 copies of a plasmid per reaction). Real-time data were collected by the 7300 System SDS Software and analyzed in Microsoft Excel. Transcript levels were normalized with rpl32 transcript levels in the same sample. Each qPCR data point is the average of three independent experiments analyzing individual biological replicates of 3 groups of 6 CA each. The primer probes sequences and accession numbers for the 6 JH biosynthetic enzymes studied, 3-hydroxy-3-methylglutaril-CoA synthase (HMCS), 3-hydroxy-3-methylglutaril-CoA reductase (HMGR), isopenyl diphosphate isomerase (IPPI), farnesyl diphthosphate synthase (FPPS), juvenile hormone acid methyltransferase (JHAMT) and methyl farnesoate epoxidase (EPOX); as well as the housekeeping gene 60S ribosomal protein L32 and TOR are included in supplemental Table 1.

2.6. RNA interference

Synthesis and microinjections of double-stranded RNA (dsRNA) were performed as described by Brandon et al. (2008). TOR and YFP (yellow fluorescent protein) target sequences for dsRNA synthesis were amplified by PCR using the TORI and YFPI primers (Supplemental Table 1). The resulting amplicons were diluted 50-fold, and 1 μl of these diluted amplicons were used as template in PCR reactions with primers containing T7 promoter sequences (TORI_T7 and YFPi_T7) (Supplemental Table 1). The products from these PCR reactions were purified using a QiAquick PCR purification kit (QiAquick sciences, Germantown, Maryland), and 1–2 μg of the purified DNA templates were used to synthesize dsRNAs with a Megascript RNAi kit (Ambion, Austin, TX). dsRNAs were precipitated using ammonium acetate/ethanol, and resuspended in ddH2O to a final concentration of 3–4 μg/μl. In each knockdown experiment, newly emerged female mosquitoes were cold anesthetized and injected intrathoracically with 1.6 μg of dsRNA using a Drummond Nanoject II microinjector and a micromanipulator.

2.7. Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software (San Diego, CA, USA). The results were expressed as means ± S.E.M. Significant differences (P < 0.05) were determined with a one tailed students t test performed in a pair wise manner or one-way ANOVA followed by a comparison of means (Turkey's test).

3. Results

3.1. Stimulation of JH synthesis by insulin

There were 2–3-fold increases in JH synthesis when CA–CC complexes dissected from 1- or 3-day old sugar-fed females were cultured for 4 h in the presence of bovine insulin (17 μM); while incubation with LY294002 (10 μM), an inhibitor of insulin signaling phosphatidylinositol 3-kinase (PI3K), resulted in 2.5–3-fold decreases in JH synthesis (Fig. 1). To further understand the role of insulin on JH synthesis we studied the effect of the insulin signaling inhibitor on the expression of six selected genes encoding enzymes; three belonging to the mevalonate pathway and three from...
the late steps in the JH biosynthetic pathway. Incubation of the CA–CC complexes dissected from 1-day old sugar-fed females in vitro with LY294002 for 6 h caused a statistically significant reduction in transcript levels for the 6 JH biosynthetic enzymes analyzed (Fig. 2), with reductions of 57% for HMGR, 52% for EPOX, 48% for FPPS, 35% for HMGR, 27% for IPP1 and 25% for JHAMT. When CA–CC dissected from 3-days old sugar fed females were treated with LY294002, we observed statistically significant reductions for transcript levels for 4 of the JH biosynthetic enzymes analyzed, with reductions of 54% for EPOX, 51% for IPP1, 42% for FPPS and 32% for HMGR. Although not statistically significant, a decrease of about 20% was observed for HMGS.

3.2. Rapamycin treatment inhibits JH synthesis

In incubations of CA–CC complexes dissected from 1- or 3-day old sugar fed females for 4 h with the TOR inhibitor rapamycin (500 nM) resulted in 2- to 2.5-fold decreases in JH synthesis (Fig. 3). Incubation of the CA–CC complexes dissected from 1-day old sugar-fed females in vitro with rapamycin for 6 h caused a statistically significant reduction in transcript levels for the 6 JH biosynthetic enzymes analyzed (Fig. 4), with reductions of 61% for EPOX, 57% for FPPS, 51% for HMGS, 43% for JHAMT 40% for HMGR, and 33% for IPP1. When CA–CC dissected from 3-day old sugar fed females were treated with rapamycin, we observed statistically significant reductions for 4 of the JH biosynthetic enzymes analyzed, with reductions of about 45–56% for IPP1, FPPS and EPOX, and 30% for JHAMT.

3.3. In vivo depletion of TOR reduced JH synthesis and decreased JH biosynthetic enzyme transcript levels

Injection of dsTOR (1.6 µg) resulted in a significant reduction of TOR mRNA (83%) when compared with dsYFP treated controls (Fig. 5A). Depletion of TOR resulted in a significant reduction in JH synthesis.

Fig. 1. Effect of insulin and LY294002 on JH synthesis. CA–CC were dissected and incubated for 4 h in the presence of 17 µM of bovine insulin, 10 µM of LY294002, or in medium alone. A) CA–CC dissected 1 day after adult eclosion. B) CA–CC dissected 3 days after adult eclosion. JH synthesis was evaluated by HPLC-FD, and it is expressed as fmol per CA per hour. Bars represent the means ± S.E.M. of the analysis of 8 individual CA–CC. Different letters above the columns indicate significant differences among treatments (one way ANOVA p < 0.05, with Tukey test of multiple comparisons).

Fig. 2. Effect of LY294002 on the mRNA levels of JH biosynthetic enzymes. CA–CC were dissected from 1-day old females and incubated for 6 h in the presence of 10 µM of LY294002, or in medium alone. Enzymes: 3-Hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), isopentenyl diphosphate isomerase (IPPI), farnesyl diphosphate synthase (FPPS), juvenile hormone acid methyltransferase (JHAMT) and methyl farnesoate epoxidase (EPOX). Transcript levels were measured by qPCR and are expressed as percentages of value measured for the control (medium alone). Bars represent the means ± S.E.M. of 3 independent biological replicates of 3 groups of 6 CA–CC. Asterisks denote significant differences from controls (unpaired t-test; *P < 0.05, **P < 0.01, ***P < 0.001).

Fig. 3. Effect of rapamycin on JH synthesis. CA–CC were dissected and incubated for 4 h in the presence of 500 nM rapamycin, or in medium alone. A) CA dissected 1 day after adult eclosion. B) CA dissected 3 days after adult eclosion. JH synthesis was evaluated by HPLC-FD, and it is expressed as fmol per CA per hour. Bars represent the means ± S.E.M. of the analysis of 8 individual CA–CC. Asterisks denote significant differences from controls (unpaired t-test; *P < 0.01, **P < 0.001).
Fig. 4. Effect of rapamycin on the mRNA levels of JH biosynthetic enzymes. CA were dissected from 1 day old females and incubated for 6 h in the presence of 500 nM rapamycin or in medium alone. Enzymes are as described in Fig. 2. Transcript levels were measured by qPCR and are expressed as percentages of value measured for the control (medium alone). Bars represent the means ± S.E.M. of 3 independent biological replicates of 6 CA. Asterisks denote significant differences from control (unpaired t-test; ***P < 0.001).

Fig. 5. Effect of RNAi silencing of TOR on JH synthesis. Newly emerged females were injected with dsYFP or dsTOR. The effect of the RNAi treatment was evaluated 4 days later. A) Efficiency of dsRNA treatment: Expression of TOR mRNA in the thorax (without legs and wings) was evaluated by qPCR and is expressed as copy number of mRNA TOR/10,000 copies of rpL32 mRNA. Bars represent the means ± S.E.M. of 3 independent biological replicates of 6 CA. **P < 0.01, ***P < 0.001. B) Effect of dsRNA treatment on JH biosynthesis. CA–CC were dissected and incubated for 4 h. JH synthesis was evaluated by HPLC-FD, and it is expressed as fmoles CA per hour. Bars represent the means ± S.E.M. of the analysis of 6 individual CA–CC. Asterisks denote significant differences from control (unpaired t-test; *P < 0.05, **P < 0.01).

Fig. 6. Effect of RNAi silencing of TOR on the mRNA levels of JH biosynthetic enzymes. Newly emerged females were injected with dsYFP or dsTOR. The effect of the RNAi treatment was evaluated 4 days later. Enzymes are as described in Fig. 2. Transcript levels were measured in the CA–CC by qPCR and are expressed as percentages of value measured for the control (YFP). Bars represent the means ± S.E.M. of 3 independent biological replicates of 3 groups of 6 CA. Asterisks denote significant differences from control (unpaired t-test; **P < 0.01, ***P < 0.001).

Fig. 7. Rapamycin hampered insulin stimulation on JH synthesis. CA–CC were dissected 3 days after adult emergence and incubated for 4 h in the presence of 17 μM of bovine insulin, 17 μM of bovine insulin + 500 nM rapamycin, 500 nM rapamycin or in medium alone. JH synthesis was evaluated by HPLC-FD, and it is expressed as fmoles CA per hour. Bars represent the means ± S.E.M. of the analysis of 6 individual CA–CC. Different letters above the columns indicate differences among treatments (one way ANOVA p < 0.05, with Tukey test of multiple comparisons).

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or absence of rapamycin (500 nM). Rapamycin impeded insulin stimulation of JH synthesis (Fig. 7).

4. Discussion

JH levels must be modulated to permit the normal progress of development and reproductive maturation in mosquitoes (Klowden, 1997). Changes in JH synthesis in female adult A. aegypti mosquito are very dynamic and nutritionally dependent (Noriega, 2004). The CA needs to adjust its synthetic activity to generate these dynamic changes (Li et al., 2003a). Adjustments in biosynthetic rates are often initiated by external alterations which set into motion changes in enzyme activities and precursor fluxes (Cascante et al., 1995; Nouzova et al., 2011). We can discriminate at least 5 distinctive periods of CA activity in pupae and adult female mosquito (early and late pupae, newly emerged, sugar fed and blood fed females). The molecular basis for JH synthesis regulation, as well as the role of endocrine regulators, might change during these 5 periods. JH synthesis is suppressed and transcript levels for the JH biosynthetic enzymes are very low in the early pupae (Nouzova et al., 2011). Subsequently, in the last 6–8 h before adult eclosion, the pupal CA becomes competent to synthesize JH, transcript levels for the biosynthetic enzymes start to rise and JH synthesis can be stimulated in vitro by supplying farnesoid acid (Nouzova et al., 2011). Afterward, the CA of the newly emerged female is fully competent to synthesize JH. For the 10–12 first hours it synthesizes relatively low levels of JH. Decapitation during these first hours after eclosion prevents increases of JH synthesis, suggesting that the brain plays a key role sensing the nutritional status and stimulating CA activity (Hernandez-Martinez et al., 2007); only when nutrients are appropriate are factors released from the brain that command the CA to synthesize enough JH to activate reproductive maturation (Caroci et al., 2004). Later, in sugar fed females, during the “resting stage” that precedes a blood-meal, the brain senses the quality of nutrition and could direct the CA to decrease JH synthetic rates causing the resorption of ovarian follicles (Clifton and Noriega, 2011); decapitation at the resting stage precludes this nutritional adjustment and causes significant increases of JH synthesis emphasizing the critical role of the brain in CA nutritional modulation (Li et al., 2004). Finally, after blood feeding; synthesis of JH is “actively” suppressed to allow the normal proceeding of vitellogenesis and egg maturation; again decapitation during this period results in a significant increase in JH synthesis (Li et al., 2004).

In insects, brain factors can stimulate (allatotropins) or inhibit (allatostatins) CA activity (Goodman and Cusson, 2012). We have described both stimulatory and inhibitory effects of brain factors in mosquitoes (Li et al., 2004; Hernandez-Martinez et al., 2007). Allatostatin-C and allatotropin are present in the brain of A. aegypti (Hernandez-Martinez et al., 2005); they both modulate JH synthesis in vitro (Li et al., 2003b, 2006) and their receptors are expressed in the CA–CC complex (Mayoral et al., 2010; Nouzova et al., 2012). The role of each of these endocrine regulators might be limited to particular periods of CA activity; insulin and/or allatotropin may well be the brain activators acting on the CA of the newly emerged female; while allatostatin-C could play a role in the nutritional modulation of JH synthesis during the “state of arrest”, as well as during the suppression of JH synthesis after a blood meal (Li et al., 2004, 2006).

We used a combination of in vitro pharmacology and in vivo genetic knockdown studies to test the hypothesis that the Insulin/TOR pathway is involved in the regulation of JH synthesis in the CA of mosquitoes. Application of bovine insulin on the mosquito CA–CC incubated in vitro caused a strong and fast stimulation on JH synthesis. Bovine insulin is effective as an analog of ILPs and acts through a conserved signaling cascade in the mosquito A. aegypti (Riehle and Brown, 1999). Insulin like peptides (ILPs) have been previously proposed as regulators of JH synthesis; Drosophila melanogaster insulin receptor (Inr) mutants had reduced JH synthesis (Tu et al., 2005) and specific silencing of the Inr in the CA of the fruit-fly completely suppressed HMGR expression and rendered a JH-deficient phenotype (Belgacem and Martin, 2007). Little is known on exactly how insulin/TOR signaling affects the activity of the CA. Systemic depletion of TOR by RNAi and rapamycin administration had inhibitory effects on JH synthesis in mosquitoes, with both treatments causing reductions in JH biosynthetic enzyme transcript levels. 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 the cockroach, Blattella germanica, systemic knock-down of TOR expression also significantly reduced HMGS and HMGR mRNA levels and inhibited JH production (Maestro et al., 2008); starvation mimicked TOR silencing effects, suggesting that TOR mediates the nutritional regulation of JH synthesis in the cockroach CA. Interestingly, systemic RNAI of forkhead-box O (FoxO), a transcriptional effector of the insulin pathway, elicited an increase of JH biosynthesis in the cockroach CA, although without modifying mRNA levels of HMGS, HMGR and EPOX (Süren-Castillo et al., 2012). The roles of FOXO and insulin in JH synthesis were also studied in C. pipiens, where the ability to enter into overwintering diapause is regulated by a shutdown in the production of JH (Spielman, 1974). Insulin signaling leads to the production of JH preventing FOXO activation and establishing a diapause phenotype. Silencing the Inr blocked JH synthesis and induced diapause, although mosquitoes could be rescued with an application of JH; suppression of FOXO had opposite effects than silencing Inr (Sim and Denlinger, 2008). Activation of the insulin signaling pathway in Drosophila is now well understood; it lead to activation of a number of kinases such as Akt and TOR via a relay of phosphorylation events; although the exact mechanism is still unclear and might occur via redundant pathways (Telemann, 2010); our studies revealed that rapamycin impeded insulin stimulation of JH synthesis in the CA of mosquitoes, confirming that TOR activity is critical for the transduction of insulin signaling. In summary, our results suggest that the insulin/TOR pathway plays an important role in the transduction of the nutritional information that regulates JH synthesis in mosquitoes. Future studies will include the use HPLC–fluorescent approaches to study the effect of manipulating the insulin/TOR pathway on precursor endogenous pools and activities of the JH biosynthetic enzymes, as well as further analysis of the role of additional insulin/TOR signaling network components on the changes in JH synthetic enzymes transcript levels.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.03.008.

References


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