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RESEARCH ARTICLE

Human IGF1 extends lifespan and enhances resistance to *Plasmodium falciparum* infection in the malaria vector *Anopheles stephensi*

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SUMMARY

The highly conserved insulin/insulin-like growth factor (IGF) signaling (IIS) pathway regulates metabolism, development, lifespan and immunity across a wide range of organisms. Previous studies have shown that human insulin ingested in the blood meal can activate mosquito IIS, resulting in attenuated lifespan and increased malaria parasite infection. Because human IGF1 is present at higher concentrations in blood than insulin and is functionally linked with lifespan and immune processes, we predicted that human IGF1 ingested in a blood meal would affect lifespan and malaria parasite infection in the mosquito *Anopheles stephensi*. Here we demonstrate that physiological levels of ingested IGF1, like insulin, can persist intact in the blood-filled midgut for up to 30 h and disseminate into the mosquito body, and that both peptides activate IIS in mosquito cells and midgut. At these same levels, ingested IGF1 alone extended average mosquito lifespan by 23% compared with controls and, more significantly, when ingested in infected blood meals, reduced the prevalence of *Plasmodium falciparum*-infected mosquitoes by >20% and parasite load by 35–50% compared with controls. Thus, the effects of ingested IGF1 on mosquito lifespan and immunity are opposite to those of ingested insulin. These results offer the first evidence that insect cells can functionally discriminate between mammalian insulin and IGF1. Further, in light of previous success in genetically targeting IIS to alter mosquito lifespan and malaria parasite transmission, this study indicates that a more complete understanding of the IIS-activating ligands in blood can be used to optimize transgenic strategies for malaria control.

Key words: insulin, insulin-like growth factor, mosquito, aging, signal transduction.

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INTRODUCTION

Malaria is responsible for a quarter of all deaths from infectious diseases and remains one of the world's great public health challenges (World Health Organization Global Malaria Program, 2011). The most vulnerable point of the malaria parasite life cycle is the transition from the human blood stream to the mosquito midgut. During this transition, hormones, growth factors and cytokines in infected blood can regulate aspects of mosquito physiology and lifespan that directly affect parasite development (Kang et al., 2008; Luckhart et al., 2003; Luckhart and Riehle, 2007; Pakpour et al., 2012; Surachetpong et al., 2009; Surachetpong et al., 2011).

In mice (Elased and Playfair, 1994) and in humans (White et al., 1983; White et al., 1987), malaria parasite infection induces hypoglycemia, which is associated with severe pathology and likelihood of a fatal outcome. In mice, hypoglycemia has been functionally linked to hyperinsulinemia (Elased and Playfair, 1996). In humans, malaria parasite infection and quinine therapy can precipitate hyperinsulinemia (White et al., 1983; Planche et al., 2005). Insulin levels in hyperinsulinemic malaria patients were 1.6×10^{-4} μmol l^{-1} on average, with the highest concentration at 4.7×10^{-4} μmol l^{-1} (White et al., 1983). These levels contrast with normal blood insulin levels, which range from 1.7×10^{-5} μmol l^{-1} at fasting to 5.9×10^{-4} μmol l^{-1} without fasting (Darby et al., 2001), indicating that blood levels of insulin can vary as much as 10- to 35-fold depending on nutrition and disease status. Normal levels of insulin-like growth factor 1 (IGF1) in human blood are much higher than levels of insulin. Concentrations range from 6 nmol l^{-1} (50 ng ml^{-1}) to 0.093 μmol l^{-1} (700 ng ml^{-1}), with median levels of 0.026 μmol l^{-1} (200 ng ml^{-1}) in healthy adults (Löfqvist et al., 2001; Renehan et al., 2003). In contrast to the rise in circulating insulin associated with malaria, IGF1 levels fall below 6 nmol l^{-1} (50 ng ml^{-1}) during severe infections with *Plasmodium falciparum* and *Plasmodium vivax* (Mizushima et al., 1994). Recent work by Umbers et al. (Umbers et al., 2011) showed a functional association between reduced serum IGF1 and the inflammation associated with placental *P. falciparum* infection.

Human insulin ingested alone in a blood meal activates endogenous insulin/IGF signaling (IIS) in the midgut of female *Anopheles stephensi* Liston 1901, an important mosquito vector of both *P. falciparum* and *P. vivax*. The blood meal is stored and digested in this organ, and it is the site of crucial phases in malaria parasite development in the mosquito host. Activation of midgut IIS can shorten mosquito lifespan and enhance susceptibility to *P. falciparum* infection in *A. stephensi* (Kang et al., 2008; Surachetpong et al., 2009). In particular, Pakpour et al. (Pakpour et al., 2012) showed that insulin-induced susceptibility is due to the sustained activation of the phosphatidylinositol 3-kinase (PI3K)/Akt branch

of the *A. stephensi* IIS, which in turn inhibits NF-κB-regulated immune gene expression. Furthermore, overexpression of Akt, a key IIS nexus protein, in the midgut of *A. stephensi* shortened lifespan and inhibited malaria parasite infection (Corby-Harris et al., 2010). Taken together, these studies indicate that activation of endogenous IIS in the mosquito midgut can dramatically affect lifespan and anti-parasite immunity.

Insulin and IGF1 are among the most important insulin-like peptides (ILPs) found in human blood. They have highly similar amino acid sequences, are structurally similar, and activate related receptor tyrosine kinases and signaling pathways. Activation of the respective receptors initiates signaling through the PI3K/Akt or the mitogen-activated protein kinase (MAPK) pathway. Key components of these pathways include p70 S6 kinase (p70S6K), the Akt-dependent forkhead transcriptional regulator FOXO and the MAPKs MEK and ERK. Signal transduction through the PI3K/Akt pathway regulates metabolism and cell survival, while the MAPK pathway affects cell proliferation. P70S6K is also activated by the target of rapamycin (TOR) signaling complex, and is an important mediator of nutrient sensing and cell growth (reviewed in Taniguchi et al., 2006). Structurally related ILPs exist in *A. stephensi* and other mosquitoes and are predicted to interact with a single receptor tyrosine kinase, the mosquito insulin receptor (MIR), which results in activation of these same conserved pathways (Marquez et al., 2011; Antonova et al., 2012).

The role of IIS in lifespan has been well studied across many taxa including nematodes, fruit flies and mice (Kenyon, 2010). Activation of IIS can decrease longevity, as we have shown for *A. stephensi* and, conversely, repression of IIS can increase lifespan in model organisms and in natural populations. Among the ILPs, IGF1 plays a crucial role in IIS-mediated lifespan regulation in mammals. For example, IGF1 levels were negatively correlated with median lifespan in 32 strains of inbred mice characterized for aging-related phenotypes (Yuan et al., 2009). Additionally, Suh et al. (Suh et al., 2008) found that partial loss-of-function mutations in the IGF1 receptor were overrepresented in centenarians, indicating that reduced signaling through the IGF1 receptor is linked to longevity in humans.

Given the pronounced conservation of human and mosquito ILP structure and IIS pathways, we sought to determine whether the effects of ingested human IGF1 on lifespan and immunity of *A. stephensi* would be analogous to or different from those of ingested insulin (Kang et al., 2008; Surachetpong et al., 2009). To this end, we first examined the persistence of ingested human IGF1 and insulin in *A. stephensi* and then determined whether IGF1 alone could activate IIS and regulate two key determinants of malaria vector capacity – longevity and susceptibility to infection – in this mosquito host.

MATERIALS AND METHODS

Reagents

Human insulin was purchased from Sigma-Aldrich (St Louis, MO, USA) and recombinant human IGF1 from R&D Systems (Minneapolis, MN, USA). Monoclonal anti-diphosphorylated ERK1/2 (Thr183, Tyr185) was obtained from Sigma-Aldrich. Anti-phospho-forkhead box O1 (FoxO1; Thr24)/FoxO3a (Thr32) antibody and anti-phospho-p70S6K (Thr412) were purchased from Millipore (Billerica, MA, USA). Anti-GAPDH antibody was purchased from Abcam (Cambridge, MA, USA). Anti-phospho Akt/PkB antibody (Ser473) was purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgG was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit F(ab')2 fragment and peroxidase-conjugated goat anti-rabbit IgG (H^+L) were purchased from Invitrogen/Life Technologies (Grand Island, NY, USA) and Pierce/Thermo Scientific (Rockford, IL, USA), respectively. The SuperSignal West Pico chemiluminescent detection kit was purchased from Pierce. All other chemicals and reagents were obtained from Sigma-Aldrich or ThermoFisher Scientific (Waltham, MA, USA). Human serum and red blood cells (RBCs) were obtained from Interstate Blood Bank (Memphis, TN, USA).

Mosquito cell culture, mosquito rearing and experimental treatments

The immortalized *A. stephensi* embryo-derived (ASE) cell line was maintained as previously described (Surachetpong et al., 2009). For *in vivo* studies, *A. stephensi* (Indian wild-type strain) were reared and maintained at 27°C and 75% humidity. All mosquito rearing and feeding protocols were approved by and in accordance with regulatory guidelines and standards set by the Institutional Animal Care and Use Committees of the University of California, Davis, and the University of Georgia.

Western blotting

For *in vivo* studies, female mosquitoes (3–5 days old) were maintained on water for 24–48 h and then allowed to feed for 30 min on reconstituted blood provided through a Hemotek Insect Feeding System (IFS; Discovery Workshops, Accrington, UK). This blood meal contained washed human RBCs and saline (10 mmol l^{-1} $NaHCO_3$, 15 mmol l^{-1} NaCl, pH 7.0) with or without recombinant human IGF1 or insulin. Midguts were dissected from 30 mosquitoes in each treatment group and processed as previously described (Surachetpong et al., 2009). Control mosquitoes were provided blood meals supplemented with an equivalent volume of IGF1 diluent [0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)].

Detection of IIS proteins followed the protocol of Surachetpong et al. (Surachetpong et al., 2009). In brief, protein lysates from cells or mosquito midguts were separated by gel electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA) and probed for proteins of interest with target-specific antibodies. Membranes were blocked in 5% dry milk/Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature, and then incubated overnight in each antibody solution. Primary and secondary antibodies were used at the following dilutions: 1:10,000 phospho-ERK (1)/1:20,000 rabbit anti-mouse IgG (2); 1:1000 phospho-FOXO (1)/1:2000 goat anti-rabbit IgG (2); 1:500 phospho-Akt (1)/1:2000 goat anti-rabbit IgG (2); 1:1000 phospho-p70S6K (1)/1:5000 goat anti-rabbit IgG (2); and 1:10,000 GAPDH (1)/1:20,000 goat anti-rabbit IgG (2).

Lifespan studies

A control and two treatment groups of *A. stephensi* females (N=300 per group, 3 days post-emergence) were fed a weekly artificial blood meal (washed human RBCs and saline, as described above) *via* a Hemotek IFS (Discovery Workshops) with: (1) 0.013 μmol l^{-1} (0.1 μg ml^{-1}) IGF1, (2) 0.133 μmol l^{-1} (1.0 μg ml^{-1}) IGF1 or (3) an equivalent volume of IGF1 diluent (0.1%BSA/PBS). Dead mosquitoes were counted three times per week, and oviposition cups were provided once a week after blood feeding. The experiment was replicated three times with separate cohorts of mosquitoes.

Preparation of radiolabeled peptides

Lyophilized human insulin (Sigma-Aldrich, 32 μg) was dissolved in 160 μl phosphate buffer (PB, pH 7.4), iodinated with ^{125}I (MP Biomedical) using chloramine T (Sigma-Aldrich), and separated from reaction byproducts by HPLC, as described in Crim et al. (Crim et al., 2002). The final ^{125}I-insulin stock concentration was 64.1 nmol l^{-1}, after adding BSA (Sigma-Aldrich, 10%, 50 μl). Lyophilized IGF1 (Novozymes, 1 mg) was dissolved in 100 μl 10 mmol l^{-1} HCl as a stock solution. For the reaction, IGF1 stock (20 μg) was diluted 1:10 in PB, radioiodinated using lactoperoxidase (Sigma-Aldrich) and separated as above. The final ^{125}I-IGF1 stock concentration was 96.4 nmol l^{-1} after addition of BSA. Radiolabeled peptides were stored at –20°C and used within 27 days after receiving the isotope.

Feeding of radiolabeled insulin and IGF1

Radiolabeled insulin (9.1 μl from stock) was added to aliquots of saline (491 μl; 30 mmol l^{-1} NaCl, 20 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} ATP, pH 7.0) and stored at −20°C for up to 30 h before use. Radiolabeled IGF1 (20.7 μl from stock) and unlabeled IGF1 (37.7 μl, 2.6 μmol l^{-1}) were added to aliquots of saline (441.6 μl) and similarly stored. Washed human RBCs (1.5 ml) were centrifuged at 1000 ***g*** for 10 min at 4°C, and 0.5 ml of the cell pellet was transferred from the bottom of the tube and added to the radiolabeled insulin or IGF1 solution immediately before feeding.

Anopheles stephensi females (50–60 individuals, 7–10 days old) kept without sucrose for 18–30 h in a humidified chamber were transferred to individual 500 ml polypropylene feeding chambers fitted with nylon mesh. Glass-jacketed feeders with a Hemotek IFS membrane (Discovery Workshops) were warmed to 37°C, set onto the nylon mesh of the feeding chambers and then filled with a mixture of saline (15 mmol l^{-1} NaCl, 10 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} ATP, pH 7.0) and RBC solution with a final concentration of 5.9×10^{-4} μmol l^{-1} insulin or 0.133 μmol l^{-1} IGF1. Females had access to the feeder for 45 min, after which engorged mosquitoes were sorted from non-engorged mosquitoes at 4°C. Feedings were performed at 11:00, 16:00 and 21:00 h to facilitate 6 h interval collections.

Electrophoresis and autoradiography

Up to 48 h post blood meal (PBM), abdomens and heads + thoraces were dissected every 6 h and separately stored in extraction solution (six heads + thoraces or six abdomens per 100 μl 40% CH_3CN, 0.1% trifluoroacetic acid in water) at −80°C. Samples were sonicated for 10 s, lyophilized and resuspended in 20 μl deionized water and 20 μl Tris-tricine gel SDS sample buffer (NuSep). They were then heated (100°C) for 5 min, centrifuged and loaded (three body part equivalents per lane) onto a Criterion 16.5% Tris-tricine gel (BioRad) along with a protein molecular weight marker mix (Kaleidoscope Polypeptide Standards, BioRad). After electrophoresis in Tris-tricine buffer for 1.5 h at 110 V, gels were dried between cellulose sheets and exposed to autoradiography film (Blue Basic Autorad Film; BioExpress, Kaysville, UT, USA) at −70°C for up to 28 days.

Radioactivity in female body parts prepared as above was quantified as counts per minute (cpm) per three body part equivalents (20 μl aliquots) on a Cobra II AutoGamma counter (Packard Instrument Company, Meriden, CT, USA). Relative amounts of insulin or IGF1 in the samples were calculated from a regression line obtained for dilutions of the radiolabeled insulin or IGF1 in RBC solutions. Feeding experiments and the above steps were replicated with three different cohorts of female mosquitoes for each of the radiolabeled peptides.

Midgut insulin receptor phosphorylation

After emergence, female *A. stephensi* were maintained on 10% sucrose for 3 days followed by water for 2 days and then given access to washed RBCs in saline alone or with human insulin (1×10^{-4} μmol l^{-1}) or human IGF1 (0.013 or 0.133 μmol l^{-1}) as above. At 0.5, 1, 3, 12 and 24 h PBM, midguts (20 per sample) were dissected into PhosphoSafe (Novagen/EMD Millipore, Billerica, MA, USA) with 4× protease inhibitor (Complete Mini; Roche Applied Science, Indianapolis, IN, USA) and transferred to 1.5 ml centrifuge tubes on ice. As a control, midguts were similarly collected from non-blood-fed mosquitoes. Following centrifugation (4°C, 5000 ***g***, 1 min), supernatant was removed and homogenization buffer (PhosphoSafe 4.75 ml; 0.25 ml of 1.0 mol l^{-1} Tris pH 7.0, 0.43 g sucrose and two tablets of Roche complete mini protease inhibitor) was added to the pelleted midguts (150 μl per sample), which were then sonicated and centrifuged (2000 ***g***, 5 min, 4°C). Supernatants were each transferred to a 1.5 ml high G-force tube on ice. Pellets were resuspended in homogenization buffer (150 μl per sample) and processed as before. This second supernatant was added to the first supernatant and centrifuged (4°C, 48,000 ***g***, 1 h). The supernatant was immediately removed, and the membrane pellet was resuspended with 20 μl of homogenization buffer (one midgut per μl). Samples were stored at −80°C.

Midgut membrane samples were resuspended in Laemmli buffer [20 μl per sample; 0.125 mol l^{-1} Tris (pH 6.8), 50% glycerol, 4% SDS, 0.02% Bromophenol Blue] and sonicated. Following incubation (30°C, 5 min) and brief centrifugation, samples (20 μl per lane) were loaded and separated on a 4–20% Tris-HCl glycine gel (BioRad Criterion; 100 V for 3 h at 4°C). Membrane proteins were transferred onto a nitrocellulose membrane (0.1 μm Protran, Whatman/GE Healthcare, Piscataway, NJ, USA; 30 V for 2 h at 4°C), which was then dried overnight. The membrane was covered with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 2 min and blocked with 2% ECL Advance blocking agent (GE Healthcare) and 2% goat serum for 2 h at 25°C. Thereafter, rabbit anti-phosphotyrosine antibody conjugated to horseradish peroxidase (Invitrogen) was added to the blocking solution (1:20,000 dilution) and incubated overnight at 4°C. Blots were rinsed for 3×20 min with TBS-T, and immunoreactive proteins were visualized with the ECL Advance kit (GE Healthcare) for image capture (GeneGnome; Syngene, Frederick, MD, USA). A total of five immunoblots were obtained from different female cohorts subjected to the same RBC feeding as described above. Each blot was processed and exposed (1–3 min) with identical conditions, and the density of immunoreactive bands corresponding to the native MIR was qualitatively assessed with GeneTools Software (Syngene). The MIR bands in the lanes with midgut membranes from non-fed females were assigned a value of one.

Malaria parasite culture and mosquito infection

Cultures of *P. falciparum* strain NF54 were grown in 10% heat-inactivated human serum and 6% washed human RBCs in RPMI 1640 with HEPES (Gibco/Invitrogen) and hypoxanthine for 15 days, or until stage V gametocytes were evident. Exflagellation rates of mature gametocytes were evaluated on the day prior to and the day of mosquito infection. Mosquitoes were fed on mature gametocyte cultures diluted with human RBCs and heat-inactivated human serum. All IGF1 treatments were added to the diluted culture just before blood feeding. Human IGF1 in 10% heat-inactivated human serum used for parasite culture ranged from 117 to 210 ng ml^{-1} (0.015–0.027 μmol l^{-1}). This serum is diluted 1:10 for parasite culture in our standard protocol, so concentrations of IGF1 in parasite culture

medium prior to recombinant IGF1 supplementation ranged from 0.0015 to 0.0027 μmol l^{-1}. Thus, IGF1 in the parasite culture medium only minimally increased the total levels of IGF1 in the experiments (see below) and the absolute concentrations of IGF1 used still bracketed the low and high ends of the normal physiological range in humans. A single source of human serum was used for all groups (controls and IGF1 treated) within each parasite infection experiment, and this serum came from non-infected individuals. Protocols involving the culture and handling of *P. falciparum* for mosquito feeding were approved and in accordance with regulatory guidelines and standards set by the Biological Safety Administrative Advisory Committee of the University of California, Davis.

For mosquito feedings, laboratory-reared 3- to 5-day-old female *A. stephensi* were maintained on water for 24–48 h prior to blood feeding. The experiment was repeated four times with separate cohorts of mosquitoes. Mosquitoes (N=125 per treatment group) were provided blood meals containing *P. falciparum* NF54-infected RBCs and treatments of 0.013 μmol l^{-1} (100 ng ml^{-1}) IGF1, 0.133 μmol l^{-1} (1.0 μg ml^{-1}) IGF1 or an equivalent volume of IGF1 diluent (0.1% BSA in PBS) *via* a Hemotek IFS (Discovery Workshops) and allowed to feed for 30 min. After 10 days, midguts from fully gravid females were dissected and stained with 0.1% mercurochrome to visualize *P. falciparum* oocysts. The mean number of oocysts per midgut (infection intensity) and the percentage of infected mosquitoes (infection prevalence; infection defined as at least one oocyst on a dissected midgut) were calculated for all dissected mosquitoes.

Plasmodium falciparum growth assays

Aliquots of *P. falciparum* NF54 culture were synchronized for 48 h as previously described (Lambros et al., 1979) and then plated in 96-well flat-bottom plates in complete RPMI 1640 with HEPES, hypoxanthine and 10% heat inactivated human serum. Parasites were treated for 48 h at 37°C with equivalent volumes of PBS and human IGF1 at concentrations ranging from 0.13 nmol l^{-1} to 1.33 μmol l^{-1}. Assays were terminated by replacing culture medium with RPMI 1640/1% formalin. Erythrocytes were stained with 10 μg ml^{-1} propidium iodide (Sigma-Aldrich) in PBS for 1 h at room temperature. Infected RBCs were counted with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Relative levels of parasite growth in response to treatment were normalized to PBS-treated controls, which were set to 100%.

Statistical analyses

Data were tested for normality using Kolmogorov–Smirnov, D'Agostino–Pearson omnibus and Shapiro-Wilk methods (GraphPad Prism 5.02, La Jolla, CA, USA). Normally distributed data were analyzed by ANOVA for overall significance and Bonferroni multiple comparison tests for pairwise comparisons. Non-normally distributed data were analyzed by Friedman's test for overall significance followed by Dunn's multiple comparison tests for pairwise comparisons. Parasite infection intensity and prevalence were analyzed to determine whether IGF1-treated mosquitoes were more resistant than controls. Data were analyzed by ANOVA to determine whether oocyst intensity in the controls differed among replicates. When no significant differences were evident, the data were pooled across replicates and analyzed by Kruskal–Wallis ANOVA to test for overall significance and Dunn's post-test for pairwise comparisons. Parasite prevalence was analyzed by Fisher's exact test to determine whether infection status differed between treatment conditions. Survival analyses were performed using the Kaplan–Meier method (Kaplan and Meier, 1958), and differences between survival curves were calculated using the Wilcoxon test (GraphPad Prism 5.02). All differences were considered to be significant at P<0.05.

RESULTS

Human IGF1 induces phosphorylation of ERK, Akt and FOXO in immortalized *A. stephensi* cells *in vitro*

To determine whether human IGF1 could activate endogenous mosquito IIS, we treated ASE cells with human IGF1 and quantified changes in the phosphorylation of Akt, FOXO and ERK (representative immunoblots in Fig. 1A). Human IGF1 induced phosphorylation of Akt (Fig. 1B), FOXO (Fig. 1C) and ERK (Fig. 1D) relative to buffer-treated (0.1% PBS/BSA) control cells. These data indicated that human IGF1 alone can activate endogenous mosquito IIS effectors *in vitro*.

Ingested human IGF1 and insulin persist intact in the mosquito for up to 30 h after feeding and disperse from the midgut

To understand the fate of ingested human insulin and IGF1, *A. stephensi* females were fed blood meals containing radiolabeled IGF1 (0.133 μmol l^{-1}) or radiolabeled insulin (5.9×10^{-4} μmol l^{-1}) alone and processed into separate abdomen and head/thorax samples every 6 h up to 48 h PBM. Autoradiographs of the body parts sampled over this period (Fig. 2A,C) revealed the ingested radiolabeled peptides and degradation products. Total radioactivity in the same sets of sampled females was quantified as fmol per body part equivalent (Fig. 2B,D).

Representative autoradiographs show that radiolabeled insulin persisted intact in the abdomen of blood-fed females for up to 24 h PBM (Fig. 2A, left panel), and intact radiolabeled IGF1 was evident for up to 30 h PBM (Fig. 2C, left panel). The abdomen contains the posterior midgut where the blood meal is stored and digested. Degradation of radiolabeled insulin in the blood filled midgut was evident at 6 h and for up to 18 h PBM as a faster-migrating band on the autoradiograph. Similarly, degraded radiolabeled IGF1 appeared as two lower bands by 6 h PBM and persisted for up to 30 h. Longer autoradiograph exposures revealed intact radiolabeled insulin and IGF1 in head/thorax samples for up to 18 h PBM (Fig. 2A,C, right panel), and no degraded forms were evident. These results indicate that the radiolabeled peptides diffuse out of the blood-filled midgut into the hemolymph and circulate into the head and thorax of female *A. stephensi*.

Quantification of total radioactivity in the body parts of females processed for the autoradiographs confirmed the persistence and dispersal of radiolabeled peptides. This method, however, does not distinguish intact and degraded forms. The amount of radiolabeled insulin in the abdomen decreased from 2 to 0.5 fmol during the first 18 h PBM (75% decrease), remained at this level until 30 h PBM and then fell to ~0.1 fmol by 48 h PBM (Fig. 2B). Radiolabeled insulin increased fourfold (0.1 to 0.4 fmol) in the head/thorax samples over 6 h PBM and remained constant thereafter (Fig. 2B). Similarly, the amount of radiolabeled IGF1 in the abdomen fell from ~400 to 100 fmol during the 24 h PBM and declined to ~50 fmol by 48 h PBM (Fig. 2D). Radiolabeled IGF1 in the head/thorax samples increased approximately threefold (25 to 75 fmol) up to 18 h PBM (Fig. 2D) and remained relatively constant thereafter.

Blood feeding rapidly induces phosphorylation of the insulin receptor in the *A. stephensi* midgut

Given the similarity in structure and signaling of human and mosquito ILPs, we asked whether ingested insulin or IGF1 at

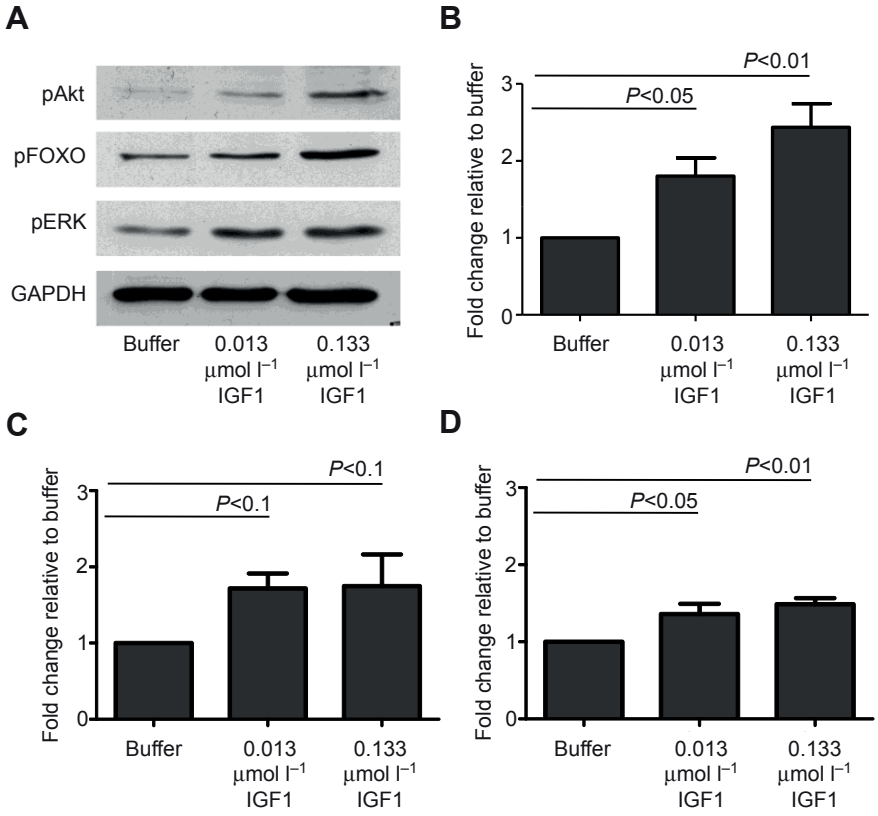

Fig. 1. Stimulation of immortalized *Anopheles stephensi* embryo-derived (ASE) cells with human insulin-like growth factor 1 (IGF1) induces phosphorylation of ERK, Akt and FOXO. ASE cells were treated with diluent buffer (0.1% BSA/PBS) as a treatment control or with 0.013 μmol l^{-1} IGF1 (0.1 μg ml^{-1}) or 0.133 μmol l^{-1} IGF1 (1.0 μg ml^{-1}) for 5 min. (A) Representative western blot showing phosphorylation of signaling proteins. (B–D) Mean ± s.e.m. fold induction of protein levels normalized first to the GAPDH loading control and then to the treatment control: (B) pAkt, (C) pFOXO and (D) pERK. Data were analyzed by ANOVA for overall significance followed by Bonferroni post-test for pairwise comparisons (α=0.05). Experiments were replicated five to seven times and *P*-values are noted on the graphs.

physiological concentrations in an artificial blood meal could induce phosphorylation of the MIR in the midgut epithelium. Representative immunoblots (Fig. 3A) showed that phosphorylation of the midgut MIR (~500 kDa) persisted for up to 3 h after females ingested washed RBCs alone or RBCs with human insulin (1.7×10^{-4} μmol l^{-1}) or IGF1 at two concentrations (0.013 or 0.133 μmol l^{-1}). Little or no phosphorylated MIR was observed in midgut samples from non-blood-fed females (results not shown). Densitometry was used to quantify phosphorylated MIR in the midgut immunoblot samples for RBC-fed mosquitoes (Fig. 3B). At 0.5 h PBM, RBCs alone increased MIR phosphorylation 3.5-fold relative to that of non-RBC-fed midguts (NF, Fig. 3A), while addition of human insulin, 0.013 μmol l^{-1} IGF1 and 0.133 μmol l^{-1} IGF1 resulted in mean increases of 3.5-, 4- and 3-fold, respectively (Fig. 3B). A progressive decline in MIR phosphorylation was evident thereafter in all midgut samples, so that by 3 h PBM mean values were not different from non-RBC-fed midguts (0 h; $P>0.1$ for all groups). No increase in MIR phosphorylation was observed at 12 and 24 h PBM (results not shown).

Ingested human IGF1 enhances phosphorylation of FOXO and p70S6K and reduces phosphorylation of ERK in the *A. stephensi* midgut

The above results showed that midgut MIR phosphorylation was significantly increased by feeding with RBCs alone or with insulin or IGF1 within less than 1 h and suggested that this robust response could obscure small differences in MIR phosphorylation – due to ingested insulin or IGF1 – that affect downstream IIS. FOXO and ERK are key mediators of the IIS downstream of the MIR, and both are phosphorylated above control levels in *A. stephensi* midguts in response to human insulin (Surachetpong and Pakpour et al., 2011). Under the same conditions, however, insulin does not induce phosphorylation of p70S6K (not shown). To compare signaling activation by IGF1 with these observations, we quantified changes in the phosphorylation of FOXO, p70S6K and ERK in midguts collected from *A. stephensi* females fed with washed RBCs alone or with washed RBCs and human IGF1 (0.013 or 0.133 μmol l^{-1}). Phosphorylation levels of FOXO and p70S6K were moderately enhanced at low levels of human IGF1 (0.013 μmol l^{-1}), but signaling protein activation in mosquitoes treated with the higher dose of IGF1 was not different from controls (Fig. 4A,B). In contrast to moderate activation of FOXO and p70S6K, both low and high IGF1 doses inhibited ERK phosphorylation in the midgut of RBC-fed *A. stephensi* ($P<0.05$; Fig. 4C). This pattern of IIS activation in the midgut differed from IGF1-induced IIS protein activation in ASE cells (Fig. 1). ASE cells are distinct metabolically from epithelial cells (Giulivi et al., 2008), but more importantly lack the tissue context of signaling controls and feedback that likely define IGF1 responsiveness *in vivo*. Therefore, in the midgut, the response to IGF1 differs from the response to insulin: IGF1 dose-dependently activates the PI3K/Akt pathway and inhibits the MEK/ERK pathway of IIS.

Provision of IGF1 in the blood meal can extend *A. stephensi* lifespan

To determine whether human IGF1 could reduce mosquito lifespan in a manner similar to human insulin (Kang et al., 2008), we monitored the survival of female *A. stephensi* provided with human IGF1 (0.013 or 0.133 μmol l^{-1}) or a buffer control (0.1% BSA/PBS) in weekly artificial blood meals. Mosquitoes fed 0.013 μmol l^{-1} IGF1 survived an average of 28 days compared with 22.6 and 23.3 days for mosquitoes provided with buffer or 0.133 μmol l^{-1} IGF1, respectively ($P<0.0001$; Table 1). Specifically, provision of 0.013 μmol l^{-1} IGF1 resulted in an average median lifespan extension of 23% when compared with controls. In two out of three experiments, the lifespans of mosquitoes treated with 0.133 μmol l^{-1} IGF1 were not different from those of controls ($P>0.05$; Table 1). Experiment 3 showed a small

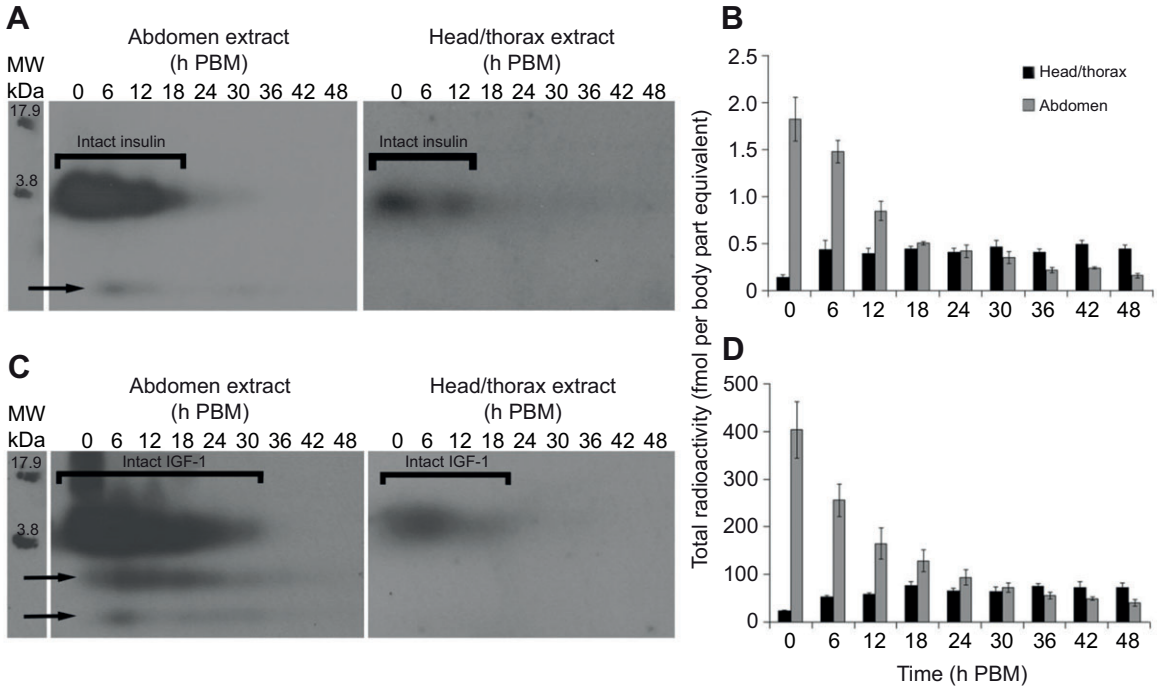

Fig. 2. Human insulin and IGF1 persist intact in the midgut during blood digestion and disperse into the body of *A. stephensi*. (A,C) Representative autoradiographs of abdomen (A, 4 day exposure; C, 0.75 day exposure) and head/thorax extract samples (A, 28 day exposure; C, 5 day exposure) processed 0 to 48 h post blood meal (PBM) containing radiolabeled insulin (A, 5.9×10^{-4} μmol l^{-1}) or IGF1 (C, 0.133 μmol l^{-1}). Three body part equivalents were loaded per lane. Insulin is the 3.8 kDa molecular weight marker (MW) in the Kaleidoscope Polypeptide Standards (BioRad) loaded in an adjacent lane. (B,D) Mean (±s.e.m.) amount of radiolabeled insulin (B) or IGF1 (D) per *A. stephensi* body part (abdomen or head/thorax) from three cohorts of experimental females.

but significant decrease in the survivorship of mosquitoes treated with 0.133 μmol l^{-1} IGF1 (P=0.045; Table 1).

Provision of IGF1 in the infected blood meal reduces *P. falciparum* development in *A. stephensi*

Although human insulin enhances *P. falciparum* development in *A. stephensi* (Surachetpong and Pakpour et al., 2011), we hypothesized that IGF1 was not likely to have the same effect on infection based on differences in midgut IIS activation following provision of insulin or IGF1. To test our hypothesis, we fed female *A. stephensi* with artificial blood meals containing *P. falciparum* and 0.0013, 0.013 or 0.133 μmol l^{-1} IGF1 or an equivalent volume of buffer (0.1% BSA/PBS) as a control. At 0.013 and 0.133 μmol l^{-1} IGF1, IGF1 treatment reduced the number of oocysts per midgut, while all three doses of IGF1 reduced the prevalence of mosquitoes infected with *P. falciparum* relative to controls (Table 2, Fig. 6). The intensity of infection was reduced from an average of 3.36 oocysts per midgut (range=0–58; N=200) in the controls to 2.21 (range=0–43; N=191)

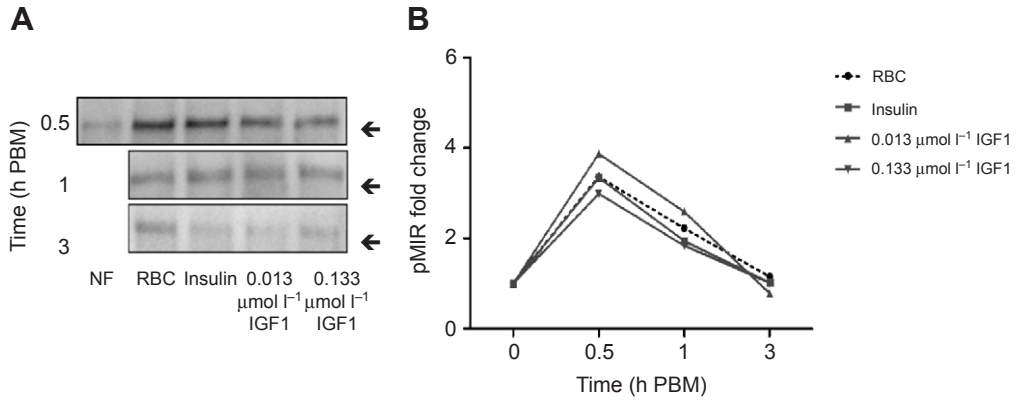

Fig. 3. Blood ingestion induces rapid phosphorylation of the insulin receptor in the *A. stephensi* midgut. Mosquitoes were fed washed red blood cells (RBCs) alone or supplemented with 1.7×10^{-4} μmol l^{-1} insulin or IGF1 at 0.013 or 0.133 μmol l^{-1}. Midguts were dissected from non-RBC-fed females (NF; shown with the 0.5 h groups) and RBC-fed females at 0.5, 1 and 3 h post blood meal (PBM) and processed for electrophoresis and western blotting. (A) Representative blots showing the phosphorylated mosquito insulin receptor (pMIR, arrows) in midgut samples from females treated as above. pMIR was detected as a single ~500 kDa immunoreactive protein. (B) Mean fold increase in pMIR in midgut samples for each treatment group at each time point relative to the non-RBC-fed group. Data were analyzed by ANOVA followed by Bonferroni multiple comparison tests for pairwise comparisons of means (α=0.05). Experiments were replicated five times with separate cohorts of mosquitoes.

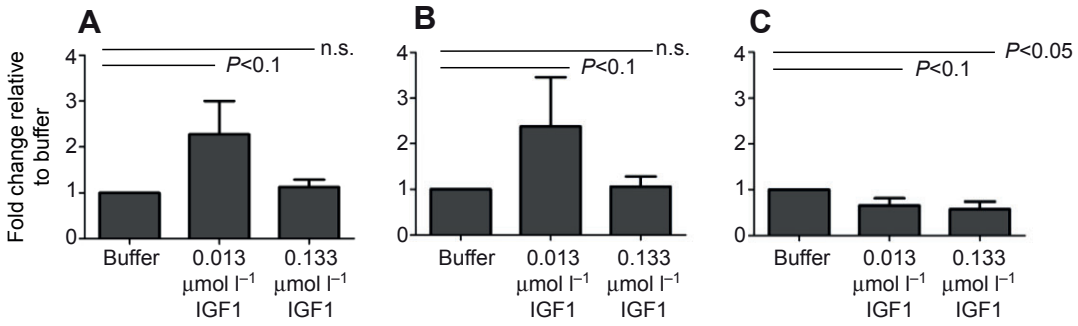

Fig. 4. Provision of human IGF1 in the blood meal induces phosphorylation of FOXO and p70S6K and inhibits phosphorylation of ERK in the *A. stephensi* midgut. Mosquitoes were fed blood meals containing 0.013 or 0.133 mol l^{-1} IGF1 or an equivalent volume of diluent buffer (0.1% BSA/PBS). Midguts were dissected at 0.5–1 h after the initiation of blood feeding. Data are represented as the mean ± s.e.m. fold induction of phospho-protein levels for p-FOXO (A), p-p70S6K (B) and p-ERK (C) quantified by densitometry and normalized first to the GAPDH loading control and then to the buffer control. Normally distributed data were analyzed by ANOVA for overall significance followed by Bonferroni multiple comparison tests for pairwise comparison of means (ERK; α=0.05). Non-normally distributed data were analyzed by Friedman's test for overall significance followed by Dunn's multiple comparison tests for pairwise comparison of means (FOXO and p70S6K; α=0.05). Experiments were replicated five times and *P*-values are displayed on the graphs.

and 1.69 oocysts (range=0–32; *N*=148) in the 0.013 µmol l^{-1} IGF1 and 0.133 µmol l^{-1} IGF1 treatments, respectively (Fig. 6A). Oocyst intensity in the 0.0013 µmol l^{-1} treatment group (range=0–59, *N*=200) was not different from the buffer control. While mean oocyst intensities did not differ significantly among groups when uninfected mosquitoes were excluded from analyses (Table 2), the percentage of mosquitoes infected with *P. falciparum* decreased from 60.5% in buffer-fed controls to 49.5, 37.7 and 38.7% in the 0.0013, 0.013 and 0.133 µmol l^{-1} IGF1 treatments, respectively (Fig. 6B).

To determine whether reduced mosquito infection was due to direct effects of IGF1 on the growth of *P. falciparum*, we examined the effects of increasing concentrations of human IGF1 on the growth of asexual stage *P. falciparum in vitro*. At all concentrations, human IGF1 had no significant effect on parasite growth (Fig. 6C), indicating that IGF1 reduced parasite infection indirectly through differential effects on signaling protein activation in the host *A. stephensi*.

DISCUSSION

Although insulin and IGF1 are structurally related to mosquito ILPs and are predicted to bind to the *A. stephensi* MIR, our findings suggest that IIS in the midgut of mosquitoes can distinguish between these two ingested peptides and evoke different physiological outcomes. Human insulin reduces lifespan and enhances parasite infection by suppressing *A. stephensi* immune processes (Kang et al., 2008; Surachetpong et al., 2009; Pakpour et al., 2012). In contrast, ingested human IGF1 can dose-dependently extend lifespan and enhance anti-parasite immune responses in *A. stephensi*.

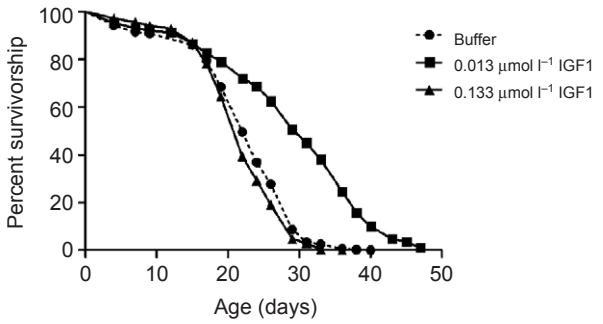

Fig. 5. Survivorship curves from Experiment 3 showing the effects of IGF1 on *A. stephensi* lifespan. Low levels of human IGF1 (0.013 µmol l^{-1} IGF1) extend *A. stephensi* lifespan, while survivorship of mosquitoes treated with higher levels of IGF1 (0.133 µmol l^{-1} IGF1) was not different from that of buffer-fed (0.1% BSA/PBS) controls. For these studies, 3- to 5-day-old *A. stephensi* females were fed weekly blood meals supplemented with physiologically low (0.013 µmol l^{-1}) or high (0.133 µmol l^{-1}) IGF1 or the IGF1 diluent buffer (0.1% BSA/PBS). A total of 300 mosquitoes were used per treatment and experiments were replicated three times with separate cohorts of mosquitoes.

We demonstrated that both insulin and IGF1 persist intact in the blood-filled midgut up to 30 h post ingestion, thus supporting the observed phosphorylation of the MIR and activation of IIS in the midgut. Both peptides also dispersed intact through the midgut into the mosquito body between 0 and 6 h after feeding, and thus may influence IIS in other tissues. Other studies have shown that much larger proteins (immunoglobulins) remain intact in the midgut of *A. stephensi* for 18 to 24 h PBM and disperse intact through the blood-filled midgut into the hemolymph (Lackie and Gavin, 1989; Vaughan et al., 1990). Following ingestion of radiolabeled insulin or IGF1, we observed a gradual decrease in radioactivity in the blood-filled midgut in the abdomen over 48 h. This decrease was likely due to diuresis, dispersal of the radiolabeled peptides into the rest of the body, enzymatic degradation in the midgut and ^{125}I-tyrosine incorporation in newly synthesized proteins in other tissues. A similar trend was observed for ^{125}I-labeled BSA fed to *A. stephensi* where ~85% of radiolabeled material was lost in diuresis products by 48 h after feeding (Schneider et al., 1986).

Our results support the conclusion that differences in the physiological roles of ingested insulin and IGF1 are not due to differences in the uptake or degradation of these peptides in the blood meal. The midgut is in direct contact with both insulin and IGF1 for the duration of blood digestion, and the peptides have ample time to influence cellular processes at the midgut epithelium. However, our results indicate that a meal of RBCs alone and with exogenous ILPs induces MIR phosphorylation and IIS within 30 min of blood feeding (Figs 3, 4), with a return to baseline phosphorylation within 3 h despite the presence of intact insulin and IGF1. Riehle and Brown (Riehle and Brown, 2002) detected the presence of MIR within cytosol and organelle samples, suggesting that the receptor in *Aedes aegypti* may be internalized in the same way as the human insulin receptor (Jensen et al., 2009; Taniguchi et al., 2006). Based on these observations, ligand-stimulated *A. stephensi* MIR may be negatively regulated by the receptor internalization or ligand desensitization that occurs during blood digestion to suppress further signaling.

Table 1. Descriptive statistics for all *Anopheles stephensi* treatment groups for Experiments 1–3

	Buffer (0.1% BSA/PBS)		0.013 μmol l^{-1} IGF1			0.133 μmol l^{-1} IGF1		
Experiment	*N*	Median	*N*	Median	*P*	*N*	Median	*P*
1	286	26	289	28	<0.0001	331	26	0.536
2	287	20	258	25	<0.0001	263	22	0.394
3	317	22	288	31	<0.0001	294	22	0.045

P-values were calculated using the Gehan–Breslow–Wilcoxon test and reflect a comparison with the matched controls at α=0.05.

In the context of strong activation of MIR by RBCs alone, insulin- and IGF1-induced changes in MIR phosphorylation were not detectable (Fig. 3). Despite this observation, artificial meals of RBCs plus insulin or IGF1 induced changes in phosphorylation of downstream signaling proteins in *A. stephensi* cells *in vivo* relative to controls. Signal transduction cascades can greatly amplify small differences in receptor phosphorylation, suggesting that IGF1-induced changes in MIR activation are reflected in larger changes in downstream IIS protein phosphorylation.

Although both human insulin and IGF1 activate endogenous mosquito IIS proteins *in vivo*, these ILPs differ in both pattern and magnitude of protein activation. In particular, human insulin consistently induced significant phosphorylation of FOXO in the *A. stephensi* midgut relative to controls (Surachetpong and Pakpour et al., 2011), whereas in the present study low IGF1 enhanced phosphorylation of FOXO, but to a lesser degree. Further, IGF1 induced phosphorylation of p70S6K in the *A. stephensi* midgut (Fig. 4), whereas insulin had no such effect (not shown). The activation of p70S6K requires sequential phosphorylation by the TOR complex, which does not activate p70S6K catalysis but rather drives a conformational change that allows access to phosphoinositide-dependent kinase, which subsequently phosphorylates p70S6K on Thr252, Ser394 and Thr412, events that are required for p70S6K catalytic activity (Weng et al., 1998). Detection of elevated levels of phosphorylated p70S6K Thr412 in the *A. stephensi* midgut after provision of RBCs and IGF1 implies that TOR-complex activation by amino acids resulting from RBC digestion (Hansen et al., 2004) cooperates with IGF1-induced phosphoinositide-dependent kinase activity (Balendran et al., 1999) to fully activate p70S6K. In further contrast with insulin, all concentrations of IGF1 reduced phosphorylation of ERK relative to control levels in the *A. stephensi* midgut. Recent work by Morcavallo et al. (Morcavallo et al., 2012) suggests how ligand binding at a single receptor could mediate such different responses: phosphorylation of the insulin receptor (IR-A) and downstream Akt and ERK varied with the affinity of the receptor for insulin, insulin analogs and IGF2. When low-affinity ligands bound the receptor, phosphorylation was reduced (compared with high-affinity ligands) and endocytosis-mediated receptor downregulation was inhibited (Morcavallo et al., 2012). This type of signaling regulation may also occur in insects. Wen et al. (Wen et al., 2010) showed that *A. aegypti* ILP3 bound to MIR in isolated ovary membranes with much higher affinity than did ILP4 or bovine insulin, indicating that the MIR varies in its affinity for endogenous ILPs as well as for mammalian insulin. Similarly, the *Drosophila melanogaster* insulin receptor can respond to different ILP ligands, as shown by the ability of DILP5 isoforms and IGFs to competitively displace human insulin from the fruit fly insulin receptor (Sajid et al., 2011).

IIS activity has been functionally associated with the regulation of lifespan (reviewed in Kenyon, 2011). Our data showed that mosquitoes treated with 0.013 μmol l^{-1} IGF1, a dose consistent with low physiological levels in normal human blood, had significantly longer lifespans than those treated with buffer or 0.133 μmol l^{-1} IGF1, a dose consistent with high physiological levels in normal human blood. Specifically, low IGF1 levels appear to generate optimal signaling through the MIR – perhaps in part *via* low dose-specific activation of FOXO and p70S6K (Fig. 4) – which resulted in extended lifespan. Reduced ILP/IGF1 receptor signaling has been linked with longevity in mice, humans, nematodes and fruit flies (Yuan et al., 2009; Suh et al., 2008; Kimura et al., 1997; Grönke et al., 2010). Grönke et al. (Grönke et al., 2010) showed that the combined knockdown of DILP2, 3, 5 and 6 in medial neurosecretory cells in *D. melanogaster* resulted in lethality, while knockdown of DILP2 alone extended lifespan, which led the authors to propose that an optimal range of IIS pathway activity can extend lifespan. Further, Biteau et al. (Biteau et al., 2010) showed that a moderate decrease in intestinal stem cell proliferation mediated by reduced IIS limited intestinal dysplasia and increased lifespan, while strong repression of intestinal stem cell proliferation *via* IIS shortened lifespan. From our studies, a low concentration of ingested IGF1 activated PI3K-mediated signaling and induced phosphorylation of p70S6K in the midgut. Given that IGF1 regulation of p70S6K is crucial to inhibition of BCL2-mediated apoptosis (Harada et al., 2001), lifetime treatment of *A. stephensi* with a low concentration of IGF1 may optimally regulate apoptosis and other processes involved in midgut maintenance and repair, resulting in longer-lived mosquitoes.

In addition to effects on lifespan, provision of human IGF1 to *A. stephensi* decreased the intensity and prevalence of *P. falciparum* infection, with high concentrations of IGF1 reducing oocyst intensity by 50% and prevalence by 37%. IGF1 reduced malaria parasite development in *A. stephensi* at the same concentrations associated with a reduction in ERK phosphorylation in the midgut. MAPK signaling proteins (including ERK) respond to stress, inflammatory mediators and growth factors, and mediate both mammalian (reviewed in Symons et al., 2006; Cargnello and Roux, 2011) and mosquito (Surachetpong et al., 2009) responses to infection.

Table 2. Summary statistics for *Plasmodium falciparum* infections

	Buffer	0.0013 μmol l^{-1} IGF1	0.013 μmol l^{-1} IGF1	0.133 μmol l^{-1} IGF1
Number of values	200	200	191	148
Minimum	0	0	0	0
Maximum	58	59	43	32
Mean, zeros included	3.365	3.965	2.209	1.689
Mean, infected only	5.562	8.175	6.029	4.310

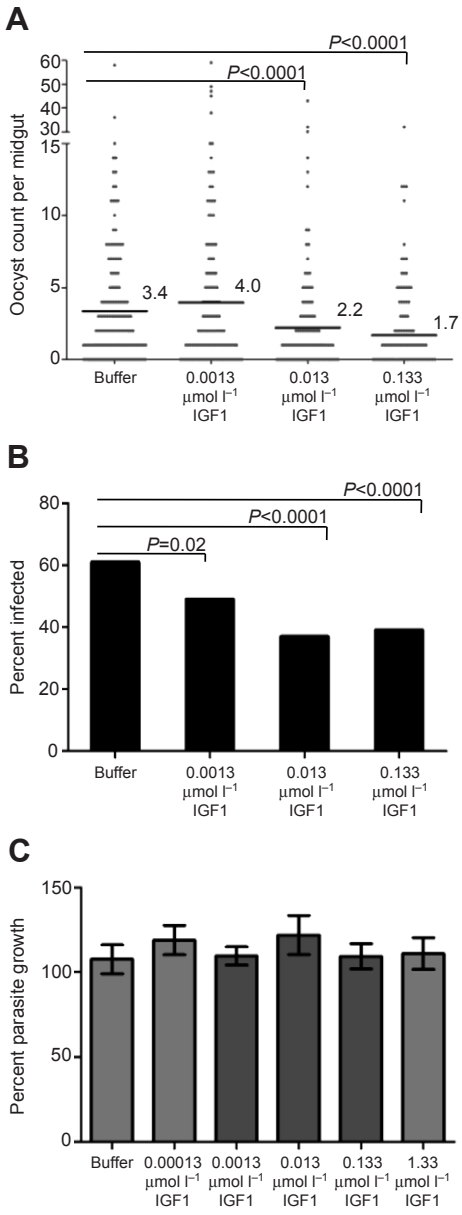

Fig. 6. Provision of human IGF1 in an infected blood meal reduces *Plasmodium falciparum* development in *A. stephensi*. Mosquitoes were fed blood meals containing malaria parasites and diluent buffer (0.1% BSA/PBS) as a control treatment, or an identical blood meal with 1.33 nmol l^{-1} to 0.133 µmol l^{-1} IGF1. Midguts were dissected and oocysts were counted at 10 days following infection. (A) IGF1 treatment decreased the number of oocysts per midgut relative to controls. This experiment was replicated four times with separate cohorts of mosquitoes and analyzed by ANOVA to determine whether the oocyst intensity in the controls differed among replicates. No differences were found, so the data were pooled across replicates and analyzed by Kruskal–Wallis to test for overall significance and Dunn's post-test for pairwise comparisons of means. (B) IGF1 treatment decreased the prevalence of malaria infection, i.e. the percent of midguts with at least one oocyst out of the total number of midguts dissected. Differences between groups were analyzed using Fisher's exact test and *P*-values are noted on the graph. (C) Human IGF1 did not affect growth of asexual-stage *P. falciparum*. Replicate cultures of *P. falciparum* NF54 were incubated with increasing concentrations of human IGF1. Relative growth is compared with the diluent buffer control, which is set at 100%. Data from three independent experiments were analyzed by ANOVA and by Bonferroni multiple comparison tests for pairwise comparison of means (α=0.05). No significant differences among treatment groups and controls were observed.

Surachetpong et al. (Surachetpong et al., 2009) showed that inhibition of ERK phosphorylation in the presence of ingested human transforming growth factor-β1 enhanced expression of the anti-parasite gene nitric oxide synthase and reduced *P. falciparum* infection prevalence, suggesting that ERK signaling inhibits mosquito innate immune processes and favors parasite development. In the context of these studies, it is possible that IGF1-dependent inhibition of ERK signaling enhances nitric oxide synthesis and other anti-parasite responses in mosquitoes. It is also interesting to note that while all concentrations of IGF1 within the normal physiological range reduced malaria parasite infection, the effects of IGF1 on lifespan are dose-dependent. Our studies suggest that differences in downstream effects of PI3K/P70S6K and MEK/ERK signaling are perhaps responsible for differences between dose-dependent effects of IGF1 on lifespan and on parasite infection.

What do our studies mean in the context of human malaria infection? Reduced serum IGF1 levels have been inversely associated with the severity of malaria infection in several studies. In children and young adults, malaria-associated hypoglycemia, an indicator of disease severity, was correlated with IGF1 levels under 50 ng ml^{-1} (mean ~6 nmol l^{-1}) (Mizushima et al., 1994). Likewise, reduced fetal and maternal IGF1 levels (mean ~40 ng ml^{-1} or 5 nmol l^{-1}) were associated with enhanced inflammation during malaria-infected pregnancies (Umbers et al., 2011). From our data, low concentrations of IGF1 (0.0013 µmol l^{-1} or 1.33 nmol l^{-1} IGF1) did not reduce parasite development in *A. stephensi*, and although infection prevalence was reduced (*P*=0.02), the magnitude of this reduction was smaller than that observed with concentrations of IGF1 (0.013 and 0.133 µmol l^{-1}) in the healthy physiological range. These observations suggest that blood containing malaria parasites and reduced IGF1 levels may be more infective to mosquitoes than blood containing parasites with IGF1 levels in a range consistent with less severe infection. Given that low levels of IGF1 have also been linked with severe malnutrition (Idohou-Dossou et al., 2003; Stephenson et al., 2000; Gomes et al., 2007), our work suggests that poor nutritional status may compound the effects of IGF1 on malaria transmission, such that efforts to improve nutrition may have an added epidemiological benefit of maintaining higher IGF1 levels that reduce parasite transmission to the mosquito host.

In summary, our results suggest that IGF1 in the normal human physiological range can extend mosquito lifespan and reduce malaria parasite infection. This work adds to a growing body of literature showing that regulation of IIS can greatly impact the capacity of mosquitoes to transmit malaria parasites. To date, we have little understanding of how clinical interventions that alter the human blood environment will alter malaria parasite transmission. This study furthers our understanding of how the molecular environment of this interface influences malaria parasite transmission and highlights the need to incorporate the dynamics of this interplay in the design of effective strategies for malaria transmission control.

LIST OF SYMBOLS AND ABBREVIATIONS

ASE	*Anopheles stephensi* embryo-derived
BSA	bovine serum albumin
IGF1	insulin-like growth factor 1
IIS	insulin/IGF-like signaling
ILP	insulin-like peptide
MAPK	mitogen-activated protein kinase
MIR	mosquito insulin receptor
PB	phosphate buffer
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinase
RBC	red blood cell
TOR	target of rapamycin

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