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Plasmodium falciparum suppresses the host immune response by inducing the synthesis of insulin-like peptides (ILPs) in the mosquito *Anopheles stephensi*

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ABSTRACT

The insulin-like peptides (ILPs) and their respective signaling and regulatory pathways are highly conserved across phyla. In invertebrates, ILPs regulate diverse physiological processes, including metabolism, reproduction, behavior, and immunity. We previously reported that blood feeding alone induced minimal changes in *ILP* expression in *Anopheles stephensi*. However, ingestion of a blood meal containing human insulin or *Plasmodium falciparum*, which can mimic insulin signaling, leads to significant increases in *ILP* expression in the head and midgut, suggesting a potential role for *As*ILPs in the regulation of *P. falciparum* sporogonic development. Here, we show that soluble *P. falciparum* products, but not LPS or zymosan, directly induced *AsILP* expression in immortalized *A. stephensi* cells *in vitro*. Further, *AsILP* expression is dependent on signaling by the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) and phosphatidylinositol 3′-kinase (PI3K)/Akt branches of the insulin/insulin-like growth factor signaling (IIS) pathway. Inhibition of *P. falciparum*-induced ILPs *in vivo* decreased parasite development through kinetically distinct effects on mosquito innate immune responses. Specifically, knockdown of *As*ILP4 induced early expression of immune effector genes (1–6 h after infection), a pattern associated with significantly reduced parasite abundance prior to invasion of the midgut epithelium. In contrast, knockdown of *As*ILP3 increased later expression of the same genes (24 h after infection), a pattern that was associated with significantly reduced oocyst development. These data suggest that *P. falciparum* parasites alter the expression of mosquito *As*ILPs to dampen the immune response and facilitate their development in the mosquito vector.

1. Introduction

The human malaria parasite, *Plasmodium falciparum*, is transmitted by mosquitoes of the genus *Anopheles* and causes over 500,000 deaths annually (WHO, 2014). Transmission of *Plasmodium* parasites requires ingestion of an infectious blood meal by a mosquito. In the mosquito midgut, ingested *Plasmodium* gametocytes undergo a series of developmental changes in the context of robust immune responses that contribute to marked losses in parasite numbers (Clayton et al., 2014; Yassine and Osta, 2010). This process is regulated in part by insulin/insulin-like growth factor signaling (IIS) and mitogen-activated protein kinase (MAPK) pathways (Corby-Harris et al., 2010; Drexler et al., 2013, 2014; Hauck et al., 2013; Horton et al., 2010; Luckhart et al., 2013; Pakpour et al., 2012; Surachetpong et al., 2009, 2010).

The regulation and function of IIS are highly conserved among vertebrate and invertebrate species (Luckhart and Riehle, 2007; Wu and Brown, 2006). In mammals, insulin biosynthesis is regulated by autocrine positive feedback that controls both insulin gene transcription and peptide secretion through activation of IIS proteins (Khoo et al., 2003; Leibiger et al., 1998, 2000; Melloul et al., 2002). Following secretion, insulin and two insulin-like growth factors (IGFs) can signal through receptor tyrosine kinase homodimers to regulate a variety of physiological processes. Additionally, mammals utilize seven other relaxin family insulin-like peptides (ILPs)

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that signal through G-protein coupled receptors (Halls et al., 2007). Varied numbers of ILPs have been identified in the fruit fly *Drosophila melanogaster* (Brogiolo et al., 2001), the nematode *Caenorhabditis elegans* (Duret et al., 1998), the silkmoth *Bombyx mori* (Mizoguchi and Okamoto, 2013), as well as the mosquitoes *Aedes aegypti* (Riehle et al., 2006), *Anopheles gambiae* (Krieger et al., 2004), and *Anopheles stephensi* (Marquez et al., 2011). Invertebrate ILPs regulate behavior (Nassel, 2012; Cator et al.,2015), metabolism (Broughton et al., 2008; Gulia-Nuss et al., 2011; Zhang et al., 2009), reproduction (Brown et al., 2008; Gulia-Nuss et al., 2011; Sim and Denlinger, 2009), aging (Evans et al., 2008a,b; Broughton et al., 2008) and immunity (Evans et al., 2008a,b; Garsin et al., 2003).

In *A. stephensi*, *ILP* expression does not change significantly with age or upon ingestion of a sugar or blood meal. However, ingestion of a *P. falciparum*-infected blood meal profoundly increased expression of *AsILP2*, *3*, *4*, and *5* in the head and midgut (Marquez et al., 2011). In support of a role for *As*ILPs in parasite infection, studies of field-collected *A. gambiae* from Mali identified a single nucleotide polymorphism (SNP) in *AgILP3* that was significantly associated with natural *P. falciparum* infection (Horton et al., 2010). We have since identified additional infection-associated SNPs in *AgILP3* and *AgILP4* that are predicted to alter protein function (unpublished data). These observations provide further evidence that mosquito ILPs – and likely *Anopheles* ILP3 and 4, in particular – contribute to the regulation of parasite development.

Strong mechanistic links between IIS and immunity have been well documented in other invertebrate species and these observations suggest that IIS and immune signaling are inversely correlated (Demas et al., 2011). That is, immune activation inhibits IIS, perhaps to allocate resources from storage and growth to the immune response, while up- and down-regulated IIS can inhibit or activate immunity to protect the host during infection and non-pathological physiological conditions, respectively. For example, activation of Toll signaling in *D. melanogaster* reduces endogenous IIS, leading to decreased nutrient storage during infection (DiAngelo et al., 2009). Similarly, *Mycobacterium marinum* infection-induced activation of Toll and IMD signaling in *D. melanogaster* resulted in a loss of metabolic stores (Dionne et al., 2006). In contrast, virulence factor-mediated increases in IIS during *Pseudomonas aeruginosa* infection in *C. elegans* suppressed anti-microbial peptide (AMP) gene expression, increasing bacterial colonization (Evans et al., 2008b), while insulin receptor *daf-2* mutants were resistant to bacterial pathogens (Garsin et al., 2003). In more recent studies, pathogen resistance in *C. elegans* was observed to occur independently of the DAF-2 pathway, resulting instead from direct pathogen activation of the IIS downstream target DAF-16, a forkhead box O (FOXO) transcripton factor (Zou et al., 2013). Our own studies affirmed that PI3K/Akt signaling is responsible for human insulin-induced repression of the innate immune responses of *A. stephensi* to *P. falciparum* infection (Pakpour et al., 2012), demonstrating that mosquito IIS regulates innate immune responses in a manner similar to that observed in *D. melanogaster* and *C. elegans*. However, little is known regarding the extent to which endogenous mosquito ILPs regulate host immunity.

Here, we build upon our previous studies demonstrating that *P. falciparum* infection can induce *AsILP* expression in the midgut to determine the mechanism and significance of this phenomenon (Marquez et al., 2011). Our results indicate that *AsILP* gene expression during *P. falciparum* infection results from parasite stimulation of IIS and not from immune activation by microbial signals that induce Toll/Immune deficiency (IMD) pathways. Induced *As*ILP3 and 4 cooperate temporally to facilitate parasite development in the mosquito by dampening NF-κB-mediated immune responses. Parasite-induced activation of IIS, therefore, suggests that *P. falciparum* has co-opted *As*ILP function in part for immune suppression in the mosquito host. Further, *As*ILP-dependent suppression of anti-parasite defenses extends our understanding of the regulation of immunity by IIS to include the activity of functionally distinct neuropeptides.

2. Results

2.1. Soluble P. falciparum products (PfPs) induced the transcription of AsILPs

We previously showed that expression levels of *AsILP2*, *3*, *4* and *5* are induced in the mosquito midgut and head following ingestion of a *P. falciparum*-infected blood meal (Marquez et al., 2011). Although *AsILP* expression was not induced in response to ingestion of blood alone, the *A. stephensi* midgut is populated with Gram-positive and Gram-negative bacteria (Djadid et al., 2011; Rani et al., 2009), including dominant Gram-negative *Asaia* sp. that can expand with blood feeding (Favia et al., 2007; Hughes et al., 2014), and with yeast symbionts (Ricci et al., 2011) that could contribute to induction of *AsILP* expression during parasite infection. To isolate and study these signals in a manner that is not possible *in vivo*, we stimulated immortalized *A. stephensi* (ASE) cells *in vitro* with lipopolysaccharide (LPS; Horton et al., 2011; Pakpour et al., 2012, 2013) and zymosan (Han et al., 1999; Heard et al., 2005) as representative Gram-negative and fungal microbe-associated molecular patterns (MAMPs), with soluble *P. falciparum* products (Pakpour et al., 2012), or with human insulin. ASE cells express *AsILP1*, *3,* and *5*, genes that are induced by physiological concentrations of human insulin and *P. falciparum* infection in *A. stephensi* (Marquez et al., 2011). However, ASE cells express only very low levels of *AsILP2* and do not express *AsILP4* (data not shown). Despite this fact, ASE signaling responses were surprisingly analogous to those in the midgut (Marquez et al., 2011). In particular, human insulin induced ASE expression of *AsILP1*, *3,* and *5* (Fig. 1A–C). While stimulation with a low concentration (3.6 parasite equivalents/cell) of soluble *P. falciparum* products (*Pf*Ps; Pakpour et al., 2012) had no effect on *AsILP* expression, stimulation with a 10-fold higher *Pf*Ps concentration (36 parasite equivalents/cell) significantly increased expression of *AsILP1*, *3,* and *5* relative to untreated controls (Fig. 1A–C). In contrast, stimulation with LPS, an activator of IMD signaling, significantly decreased expression of *AsILP1* and *AsILP3* relative to control (Fig. 1A and B), while zymosan, an activator of Toll signaling, had no significant effect on *AsILP* expression, suggesting that bacterial and fungal MAMPs that induce Toll/IMD signaling do not contribute to induction of *AsILP* expression during *P. falciparum* infection in *A. stephensi*.

2.2. AsILP expression was dependent on both IIS-associated MEK/ERK and PI3K/Akt signaling

We previously observed that *P. falciparum* induction of *nitric oxide synthase* (*NOS*) expression in ASE cells (Lim et al., 2005) and in the *A. stephensi* midgut is dependent on activation of both MEK/ERK (Surachetpong et al., 2009) and PI3K/Akt (Corby-Harris et al., 2010; Luckhart et al., 2013) signaling. Here we have demonstrated that *Pf*Ps and human insulin induce *AsILP* transcription and that this signaling is distinct from pathways activated by LPS (Imd) and zymosan (Toll) that decrease or do not induce *AsILP* expression, respectively (Fig. 1). Thus, we reasoned that expression of *AsILPs* in *A. stephensi* could be regulated by MEK/ERK and PI3K/Akt signaling. Our analyses revealed that stimulation of ASE cells with *Pf*Ps led to increased phosphorylation of the MEK/ERK branch of IIS (Fig. 2A), with statistically significant increases in phosphorylated ERK levels