Induction of Nitric Oxide Synthase in Anopheles stephensi by Plasmodium falciparum: Mechanism of Signaling and the Role of Parasite Glycosylphosphatidylinositol

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Malaria parasite (Plasmodium spp.) infection in the mosquito Anopheles stephensi induces significant expression of A. stephensi nitric oxide synthase (AsNOS) in the midgut epithelium as early as 6 h postinfection and intermittently thereafter. This induction results in the synthesis of inflammatory levels of nitric oxide (NO) in the blood-filled midgut that adversely impact parasite development. In mammals, P. falciparum glycosylphosphatidylinositolos (PfGPIs) can induce NOS expression in immune and endothelial cells and are sufficient to reproduce the major effects of parasite infection. These effects are mediated in part by mimicry of insulin signaling by PfGPIs. In this study, we demonstrate that PfGPIs can induce AsNOS expression in A. stephensi cells in vitro and in the midgut epithelium in vivo. Signaling by P. falciparum merozoites and PfGPIs is mediated through A. stephensi Akt/protein kinase B and a pathway involving DSOR1, a mitogen-activated protein kinase kinase, and an extracellular signal-regulated kinase. However, despite the involvement of kinases that are also associated with insulin signaling in A. stephensi cells, signaling by P. falciparum and by PfGPIs is distinctively different from signaling by insulin. Therefore, although mimicry of insulin by PfGPIs appears to be restricted to mammalian hosts of P. falciparum, the conservation of PfGPIs as a prominent parasite-derived signal of innate immunity can now be extended to include Anopheles mosquitoes, indicating that parasite signaling of innate immunity is conserved in mosquito and mammalian cells.

Anopheles stephensi, a primary vector of Plasmodium spp. in India and the Middle East, limits malaria parasite development with the inducible synthesis of nitric oxide (NO) (34) catalyzed by A. stephensi NO synthase (AsNOS) (32, 33). Induction of AsNOS expression is proportional to the intensity of parasite infection and is detectable in the midgut by 6 h postinfection (15, 31). Early induction is critical to inhibition of parasite development: dietary provision of the pan-NOS inhibitor N-nitro-L-arginine, with a half life in blood of 3 to 6 h (13), resulted in significantly higher parasite infection intensities than did the inactive enantiomer N-nitro-D-arginine (34).

The NO-mediated defense of A. stephensi is analogous to mammalian NO-mediated inactivation of liver-invading sporozoites and blood-stage gametocytes (36, 42), indicating that mosquitoes and mammals share a conserved antiparasite defense. The activation of mammalian immune effectors, including inflammatory cytokines, adhesion molecules and iNOS, has been attributed to parasite GPIs (reviewed in (22) and to hemozoin (27, 38, 55). In general, GPIs consist of a conserved ethanolamine phosphate-trimannosylglucosaminyl glycan core attached to phosphatidylinositol. GPIs are ubiquitous in eukaryotic cells, where their primary function is to anchor proteins to the cell membrane. In the case of P. falciparum GPIs (PfGPIs), key structural features include a terminal fourth mannose, variable fatty acyl substituents with unsaturated acyl residue on sn-2 position on glycerol, and C16:0 acyl moiety on C-2 of inositol (22). Compared to animal cells, parasites express GPIs at levels severalfold higher than are required for protein-anchoring (20). A number of studies during the past decade have shown that GPIs of various pathogenic parasites, including Plasmodium, Trypanosoma, and Leishmania species, are biologically active. For example, parasite GPIs can induce the production of proinflammatory cytokines and NO (49, 63).

From the point of view of the host, these innate immune responses represent a first line of defense for recognition and elimination of parasites through responses that are toxic to invading microorganisms.

Early studies revealed that PfGPIs could induce lipogenesis and glucose oxidation in rat adipocytes and that injection of PfGPIs into mice could induce hypoglycemia (52). These observations led to the hypothesis that PfGPIs were insulin-mimetic. Subsequently, it was demonstrated that malaria parasite GPIs exhibited signaling characteristics of the insulin second messenger phosphoinositolglycan (PIG) (7), which is released from host cell GPI by insulin stimulation of phosphatidylinositol-dependent phospholipase activity. Although no additional studies have examined the insulin-like signaling behavior of PfGPIs in detail, studies with synthetic insulin-mimetic PIGs, developed for treatment of insulin-resistant diabetes, provide relevant insight into parasite GPI signaling. In adipocytes, the insulin-mimetic PIGs bypass insulin receptor (INR) activation and instead interact with an unidentified cell surface protein to
induce tyrosine phosphorylation of mammalian insulin receptor substrates (IRSs), including IRS-1 and IRS-3 (21, 39). PIG-dependent IRS phosphorylation is then followed by signaling through the two major insulin signaling pathways (Fig. 1) involving phosphatidylinositol 3-kinase (PI3-K), Akt/PKB, and MEK/ERK (21).

Differential activation of mosquito immune genes by bacteria and Plasmodium spp. (18, 19, 34, 44) indicates a degree of immune recognition that may be based in part on activation of host pathways by parasite GPs. Available data on well-known GPI-linked parasite proteins suggest that GPs derived from both asexual and sexual stages would be available to signal induction of AsNOS expression in A. stephensi from bloodmeal ingestion through sporogonic development (16, 24, 37, 51). In addition, recent work suggests that relevant signaling pathways in mosquito cells could transduce signals from PIGGPIs for AsNOS induction. Insulin signaling gene products orthologous to those in Drosophila melanogaster (11, 48) have been described from Aedes aegypti (45, 46) and from Anopheles gambiae (47). Insulin signal transduction can induce iNOS expression in mammalian cells, revealing a connection between insulin signaling and inflammation (3). In Caenorhabditis elegans, the insulin signaling pathway upregulates antimicrobial response genes, suggesting that the link between insulin signaling and host defense has been conserved through evolution (40). In this study, we show that P. falciparum GPs can induce AsNOS expression in vitro and in vivo by activating kinases associated with insulin signaling, indicating that both signaling by GPs and functional relevance of GPs to innate immunity are evolutionarily conserved.

MATERIALS AND METHODS

Materials. Chemicals, antisera, and other reagents were purchased from the following companies: human serum and human red blood cells from Continental Services Group; RPMI 1640, Trizol reagent, and Topo TA cloning kit from Invitrogen Life Technologies; minimal essential medium (MEM) from Cellgro; hydroxy-2-naphthalenemethylphosphonic acid-Trisacetyloxymethyl ester and genistein from Calbiochem; LY294002, wortmannin, PD98059, human insulin, and monoclonal mouse anti-phospho-ERK antisera from Sigma-Aldrich; bovine serum albumin from Fisher Scientific; polyclonal rabbit anti-phospho-INS antisera, polyclonal rabbit anti-phospho-PKB antisera, polyclonal rabbit anti-phospho-JNK/SAPK antisera, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) from Biosource International; HRP-conjugated anti-mouse IgG and SuperSignal West Pico chemiluminescent detection kit from Pierce; lactate reagent from Trinity Biotech; Moloney murine leukemia virus reverse transcriptase from Applied Biosystems; T7 RNA polymerase and MEGAscribe T7 transcription kit from Ambion; Effective Transfection Reagent from OIAGEN; and protease inhibitor cocktail from Roche Diagnostics.

Isolation of P. falciparum merozoites, purification of PIGGPIs, and stimulation of ASE cells. For preparation of merozoites, P. falciparum (FCR-3 strain) was cultured to 30% parasitemia and incubated at 0.2% hematocrit as described (41) to prevent reinvasion of merozoites. The culture was centrifuged at 900 rpm at 4°C 5 min to ensure that the majority of insects were engorged. Midgeats of blood-fed mosquitoes during subsequent centrifugation of the supernatant at 1,800 rpm, approximately 50% of the total merozoites and the remaining infected and uninfected red blood cells formed a layered pellet. The top layer of merozoites was carefully aspirated. The supernatant was then centrifuged at 3,600 rpm to pellet the remaining merozoites. Collected merozoites were combined and washed with endotoxin-free incomplete RPMI medium and stored at −80°C. Protein-free PIGGPIs were isolated as described previously and purified by high-pressure liquid chromatography (28, 41). All preparations of PIGGPIs used in our work were tested for endotoxin (28).

Anopheles stephensi rearing, infection with P. falciparum, and provision of PIGGPIs by artificial bloodmeal. Anopheles stephensi Liston were reared at 27°C and 75% relative humidity. Use of mice and hamsters as bloodmeal sources in the rearing of A. stephensi is in compliance with all federal guidelines and institutional policies. For infection with P. falciparum, 4- to 5-day-old mosquitoes were allowed to feed on an artificial bloodmeal containing cultured D. melanogaster (Ag) (47) and D. melanogaster (Dm) (11, 48), and provision of PIGGPIs (Dm) (11, 48), indicating...

FIG. 1. Generalized insulin signal transduction pathways. Selected orthologous receptor and pathway elements from human (Hs), D. melanogaster (Dm) (11, 48), A. aegypti (Aa) (45, 46) and A. gambiae (Ag) (47) are indicated.
FIG. 2. *AsNOS* expression is induced by *P. falciparum* merozoites and PIGPIs. (A) ASE cells were stimulated with 15.6 merozoites per cell (equivalent to 0.25 μM GPIs; *n* = 3), with 1.56 merozoites per cell (equivalent to 0.025 μM GPIs; *n* = 2) or incomplete medium as a control for 48 h. (B) ASE cells were stimulated with 2.5 μM (*n* = 3), 0.25 μM (*n* = 5), or 0.025 μM PIGPIs (*n* = 1) in 80% ethanol or with an equivalent volume of 80% ethanol as a control for 48 h. (C) Midguts were dissected from *A. stephensi* immediately after (0 h) and from 1–48 h post-blood meal after PIGPI or control feeding for analysis of *AsNOS* expression. Data were derived from two replicates of experimental and control feeds with two separate cohorts of *A. stephensi*. In panels A to C, *AsNOS* expression levels were divided by control expression levels to show relative induction. Values represent means ± SE. Data within each treatment or time point versus the control were analyzed using the Student *t* test; *P* values are shown.

Stimulation of *A. stephensi* cells. Immortalized, embryo-derived *A. stephensi* cell lines, ASE (29, 31) and MSQ43 (generously provided by the Department of Entomology, Water Reed Army Institute of Research (31), were cultured in modified MEM containing 5% heat-inactivated fetal bovine serum (ES medium) at 28°C under 5% CO₂. For stimulation, 1 × 10⁶ cells were seeded in 96-well plates and allowed to grow overnight. Cells were stimulated with merozoites, incomplete medium, human insulin, or HEPES buffer. For kinase inhibition studies, cells were pretreated with inhibitors or diluents of inhibitors as controls for 30 to 60 min and then stimulated. For Western blot analyses, cells were harvested at 5 to 30 min after stimulation, lysed in buffer described above and prepared for analysis. At 48 h after stimulation, RNA isolation and *AsNOS* expression analyses were performed as described above.

Western blot analyses. Lysates prepared from stimulated cells and midgut tissues were centrifuged at 10,000 rpm at 4°C for 10 min to remove insoluble material. Supernatant proteins were electrophoretically separated by SDS-PAGE and then transferred to nitrocellulose membrane using a semidry blotter (Bio-Rad). The membranes were blocked with Tris-buffered saline (pH 7.4; TBS) containing 3% bovine serum albumin. After washing with TBS containing 0.1% Tween 20, the membranes were incubated with a 1:3,000 dilution of polyclonal rabbit anti-phospho-INR antisera, a 1:1,000 dilution of polyclonal rabbit anti-phospho-JunOSA antisera, a 1:1,000 dilution of polyclonal rabbit anti-phospho-PKB antisera, or a 1:10,000 dilution of monoclonal mouse anti-phospho-ERK antisera for 2 h at room temperature. The INR antisera recognizes three phosphotyrosine residues within the activation loop of the receptor, while the PKB antisera recognizes a threonine phosphorylated by PDK1 and the ERK and JNK/SAPK antisera recognize bisphosphorylated (pT/pY) ERK and bisphosphorylated (pT/pY) JNK/SAPK, respectively. The sequences of peptides used to generate these antisera are 100% conserved with predicted amino acid sequences among orthologous proteins from mammals, *D. melanogaster* and/or other mosquito species, and, as such, were expected to recognize relevant *A. stephensi* proteins. Membranes were then washed and incubated with a 1:250,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or a 1:5,000 dilution of HRP-conjugated anti-mouse IgG. Peroxidase activity was detected with the SuperSignal West Pico chemiluminescent detection kit. For JNK/SAPK Western blot, signal intensities were measured using a GS-800 calibrated densitometer (Bio-Rad) and normalized against untreated, control cells.

Measurement of lactate release. ASE cells (1 × 10⁶ per well in 96-well plates) were stimulated with human insulin, PIGPIs, or *P. falciparum* merozoites for 4 h. Cells stimulated with HEPES buffer, 80% ethanol, or incomplete medium were used as controls. Culture media were collected after 4 h and lactate level was measured as described (8) using lactate reagent.

Expression of INR, DSOR1, and Akt/PKB gene orthologs in the *A. stephensi* midgut. Total RNA was isolated from 10 to 15 *P. falciparum* infected and uninfected *A. stephensi* midguts at 24 h post-blood meal and from ASE cells as described. First strand cDNA was synthesized using MuLV reverse transcriptase. Fragments of *A. stephensi* INR, DSOR1, and Akt/PKB genes were amplified with degenerate primers: INR forward 5’ GGCTGTTNGGTATGGTNTTA 3’; INR reverse 5’ CGTTCATTACNCCACGTC 3’; DSOR1 forward 5’ CGGANACGCGGANATAC 3’; DSOR1 reverse 5’ TCCANGGGCGNTTCTACAG 3’; Akt/PKB forward 5’ TTCACCCTCTCATCCGGGG 3’; Akt/PKB reverse 5’ ATCATCTCGATCTAGCNAGCC 3’. PCR products were cloned into Topo TA plasmid. Double-stranded, partial sequences of *A. stephensi* INR, DSOR1, and Akt/PKB gene orthologs were deposited in GenBank and these sequences were used to design gene-specific primers for RT-PCR.

Conditions for RT-PCR analyses of the *A. stephensi* genes were as follows. INR: forward primer 5’ GGGTGGTTGGGTATGGTNTTA 3’ and reverse primer 5’ CGTTCATTACNCCACGTC 3’, 1 cycle of 95°C for 10 min, 35 cycles of 94°C 30 s, 53°C 30 s, 72°C 1 min, and 1 cycle of 72°C 10 min. DSOR1: forward primer 5’ CGGAGAGCCGCAAATAC 3’ and reverse primer 5’ TTCTATG GCGGCGGTTCTACAG 3’, 1 cycle of 95°C 10 min, 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s, and 1 cycle of 72°C 10 min. Akt/PKB: forward primer 5’ TTCACCCTCTCATCCGGGG 3’; Akt/PKB reverse 5’ ATCATCTCGATCTAGCNAGCC 3’. PCR products were cloned into Topo TA plasmid. Double-stranded, partial sequences of *A. stephensi* INR, DSOR1, and Akt/PKB gene orthologs were deposited in GenBank and these sequences were used to design gene-specific primers for RT-PCR.

Suppression of Akt/PKB and DSOR1 mRNA levels in MSQ43 cells by RNA interference (RNAi) and analyses of *AsNOS* induction. To produce templates for double-stranded RNA synthesis, 858bp and 500bp of *A. stephensi* Akt/PKB and DSOR1 were amplified from plasmid clones by PCR. Sense and antisense ssRNA were synthesized using the Lig’nScribe kit and the MEGAscript T7 transcription kit. Double-stranded RNAs of *A. stephensi* Akt/PKB and DSOR1 were produced as described (30). For each transfection, 2.5 × 10⁶ MSQ43 cells in 10 ml ES medium were transfected with 2 μg of double-stranded RNA using Effectene Transfection Reagent; control cells were treated in an identical manner but without double-stranded RNA (e.g., mock transfection). To examine the reduction of mRNA levels of Akt/PKB and DSOR1 in transfected MSQ43 cells, total RNA was isolated from 6 h to 5 d posttransfection and RT-PCR was performed.
as described above. For stimulation experiments, culture medium was removed from MSQ43 cells at 12 h posttransfection and 1 × 10⁶ MSQ43 cells were resuspended in 96-well plates. Cells were allowed to stabilize for 6 h and then stimulated with *P. falciparum* merozoites, incomplete medium, human insulin, or HEPES buffer for 48 h. Following stimulation, *AsNOS* expression was measured by quantitative RT-PCR as described above.

DNA sequencing and analysis. DNA sequencing was carried out by using a dideoxy dye termination method on an ABI sequencer (Perkin-Elmer Cetus) by UC Davis Sequencing. Nucleotide sequences were compared against standard databases and deposited in GenBank for *AsNOS* (AY697415), *AsDSOR1* (AY697414) and *AsAkt/PKB* (AY697413).

Statistical analyses. Data from replicated analyses are represented as means ± standard errors (SEs) and were analyzed using the Student’s t-test. P values are shown in graphical representations of the data.

**RESULTS**

*AsNOS* expression in *A. stephensi* cells is induced by *P. falciparum* merozoites and PIGPIs. To identify the *P. falciparum* factor(s) responsible for *AsNOS* induction, we established an in vitro system using *A. stephensi* embryo-derived ASE and MSQ43 cell lines, tractable and proven models for *AsNOS* induction (31). *Anopheles stephensi* cells were stimulated with *P. falciparum* merozoites at a ratio of 15.6 or 1.56 merozoites per cell. These parasite to host cell ratios are comparable to natural infection in vivo. Specifically, ingestion of 1 to 2 μl of blood by *A. stephensi* from a host with a 10% parasitemia, typical in peripheral blood samples from life-threatening *P. falciparum* infections (5), would lead to midgut infections of 250 to 550 parasites per midgut epithelial cell. Our in vitro assays of 15.6 or 1.56 merozoites per mosquito cell would be typical of mosquito feeding on hosts with lower (0.25 to 0.5%) parasitemias (35). At 48 h after treatment, 15.6 and 1.56 merozoites per ASE cell induced *AsNOS* expression >3-fold (*P* = 0.08) and >1.5-fold (*P* = 0.05), respectively, compared to medium-treated controls (Fig. 2A). Merozoites induced similar levels of *AsNOS* expression in MSQ43 cells (see below; Fig. 8D and 9D). These results are consistent with levels of *AsNOS* expression induced by natural parasite infection in vivo (31) and demonstrated that whole parasites contained *AsNOS*-inducing factors that were recognized by *A. stephensi* cells.

To determine whether PIGPIs could induce *AsNOS* expression, ASE cells were stimulated with PIGPIs at concentrations equivalent to ingestion by *A. stephensi* of ~40,000 or 400,000 parasites (1% or 10% parasitemia [5]). Given that each malaria parasite contributes 10⁷ GPIs (20), meals of 40,000 or 400,000 parasites would contain ~0.32 μM or 3.2 μM GPIs. Based on these calculations, we selected 0.025 to 2.5 μM PIGPIs for stimulation. Treatment with 0.25 μM and 2.5 μM PIGPIs induced *AsNOS* expression in ASE cells 1.7-fold (*P* = 0.01; Fig. 2B) and 5.2-fold (*P* = 0.03; Fig. 2B), respectively, compared to controls, indicating that biologically relevant levels of PIGPIs could induce *AsNOS* expression in our in vitro model system. Our results also suggested that *P. falciparum* components other than GPIs could induce *AsNOS* expression since 15.6 and 1.56 merozoites per ASE cell (equivalent to 0.25 μM and 0.025 μM PIGPIs, respectively) induced *AsNOS* expression 3-fold and 1.5-fold, respectively, (Fig. 2A), whereas 0.25 μM PIGPIs induced *AsNOS* expression only 1.7-fold and 0.025 μM PIGPIs did not induce *AsNOS* expression relative to controls (Fig. 2B).

*AsNOS* Expression in the *A. stephensi* midgut epithelium is induced by PIGPIs. To determine whether PIGPIs could function as an *AsNOS*-inducing ligand in vivo, we provided 2.5 μM PIGPIs in artificial bloodmeals to two separate cohorts of *A. stephensi*. Age-matched mosquitoes fed equivalent bloodmeals with only 80% ethanol added were used as controls. At various times after feeding, samples of total RNA from dissected midguts were analyzed for *AsNOS* expression by quantitative RT-PCR. *AsNOS* expression in midguts from PIGPI-treated *A. stephensi* was induced 1.4-fold (*P* = 0.05), >2.5-fold (*P* = 0.1), and >2-fold (*P* = 0.03, *P* = 0.01) relative to controls at 0 h (immediately after feeding) and at 1 h, 24 h and 48 h post-blood meal (Fig. 2C), indicating that PIGPIs signal *AsNOS* induction in vivo.

Putative INR-, DSOR1-, and Akt/PKB-encoding genes are expressed in *A. stephensi* cell lines and in the midgut. Based on previous observations that PIGPIs are insulin-mimetic (7, 52) and that insulin signaling can induce *NOS* expression in mammalian cells (3), we hypothesized that parasite GPIs signal *AsNOS* induction through pathways involving PI3-K, Akt/PKB and MEK/ERK. Initially, to establish the presence of these signaling molecules in mosquitoes, we characterized expression of *A. stephensi* genes encoding a predicted INR, the MEK homolog DSOR1, and Akt/PKB (Fig. 3). In nonquantitative RT-PCR assays, we determined that all three genes are expressed in the *A. stephensi* midgut, in the presence and absence of *P. falciparum* infection, and in the ASE cell line (Fig. 4). We also determined that ASE cells and the midgut epithelium are responsive to human insulin. Specifically, 1.7 μM insulin induced *AsNOS* expression in ASE cells maximally to 2.2-fold relative to controls at 22 h after treatment (Fig. 5A and B), while the same insulin concentration induced *AsNOS* expression in the midgut to 1.9-fold at 6 h and to 3.6-fold at 36 h postfeeding relative to controls (not shown).

*Plasmodium falciparum* merozoites signal *A. stephensi* cells through non-INR protein tyrosine kinase. To determine whether *P. falciparum* signaled *AsNOS* induction through activation of an *A. stephensi* INR, ASE cells were pretreated with *HNMPA-(AM)₃*, an INR tyrosine kinase inhibitor, prior to stimulation. In these and subsequent inhibitor assays, human insulin was used as a standard to verify involvement of insulin signaling pathway components. *Plasmodium falciparum* merozoites were used to stimulate ASE cells to account for potential involvement of signaling factor(s) other than PIGPIs. Pretreatment of ASE cells with 0.1 μM or 1 μM *HNMPA-(AM)₃* led to, respectively, 45% (*P* = 0.02) and 32% (*P* = 0.02) decreases in insulin-induced *AsNOS* expression and 92% (*P* = 0.0002) and 84% (*P* = 0.0001) decreases in merozoite-induced *AsNOS* expression relative to controls (Fig. 6A). These results suggested that INR activation is necessary for both insulin and parasite induction of *AsNOS*. In *A. aegypti*, *HNMPA-(AM)₃*, inhibited bovine insulin signaling in ovaries, although a 90% reduction in signaling required a much higher concentration (1 mM) of *HNMPA-(AM)₃* than was used in our assays (46). In our assays with *HNMPA-(AM)₃* and other inhibitors (see below), we noted an unexpected pattern of higher *AsNOS* induction levels at higher inhibitor concentrations. We determined that treatment of ASE cells with high concentration of inhibitors induced phosphorylation of a putative JNK/SAPK (Fig. 7), a signaling protein whose activation has been associ-
FIG. 3. Alignment of predicted amino acid sequences of AsINR, AsDSOR1, and AsAkt/PKB with predicted orthologous sequences from A. gambiae, A. aegypti, and D. melanogaster. Conserved amino acids are represented in bold type. (A) The predicted amino acid sequence of AsINR gene fragment (AY697415) was aligned with A. gambiae INR (XP_320130), A. aegypti INR (AAB17094), and D. melanogaster INR (NP_524436). (B) The predicted amino acid sequence of AsDSOR1 gene fragment (AY697414) was aligned with A. gambiae DSOR1 (XP_322064) and D. melanogaster DSOR1 (NP_511098). (C) The predicted amino acid sequence of AsAkt/PKB gene fragment (AY697413) was aligned with A. gambiae Akt/PKB (EAA03708), A. aegypti Akt/PKB (AAP37655) and D. melanogaster Akt/PKB (CAA81204). Nucleotide sequences encoding overlined amino acid sequences were used to design gene specific primers for RNAi.
A representative figure of RT-PCR results is shown. Amplifications from cDNA and RNA prepared from ASE cells, and midgut tissue 24 h after an uninfected bloodmeal (Uninf midgut), amplifications from cDNA and RNA prepared from \textit{A. stephensi} midgut tissue 24 h postinfection with \textit{P. falciparum} (Inf midgut), amplifications from cDNA and RNA prepared from \textit{A. stephensi} midgut tissue 24 h after an uninfected bloodmeal (Uninf midgut), amplifications from cDNA and RNA prepared from ASE cells, and control amplifications without added cDNA or RNA (no-template control, NTC). A representative figure of RT-PCR results is shown.

FIG. 5. \textit{AsNOS} expression is induced maximally in ASE cells by 1.7 \textmu M human insulin at 48 h after stimulation. (A) ASE cells were stimulated with various concentrations of human insulin in HEPES buffer \((n = 3 \text{ for } 0.17 \text{ \textmu M}, n = 7 \text{ for } 0.85 \text{ \textmu M}, n = 8 \text{ for } 1.7 \text{ \textmu M}, n = 3 \text{ for } 3.4 \text{ \textmu M}, n = 4 \text{ for } 17 \text{ \textmu M})\) or HEPES buffer only as a control for 48 h. (B) ASE cells were stimulated with 1.7 \textmu M human insulin or HEPES buffer for 2–48 h \((n = 4 \text{ for } 2 \text{ h}, n = 4 \text{ for } 6 \text{ h}, n = 4 \text{ for } 12 \text{ h}, n = 4 \text{ for } 24 \text{ h}, n = 4 \text{ for } 36 \text{ h}, n = 9 \text{ for } 48 \text{ h})\). For panels A and B, \textit{AsNOS} expression levels were measured by quantitative RT-PCR and were divided by control expression levels to show relative induction. Values represent means ± SEs.
inhibitor (AM)3, an inhibitor of INR activation, dissolved in dimethyl sulfoxide as a mock pretreatment (0 μM) or with 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium (n = 2 for both inhibitor concentrations) for 48 h prior to analysis of AsNOS expression. (B) Anti-phospho-INR Western blot of ASE cells stimulated with 1.7 μM human insulin in HEPES buffer (n = 7 for 0.1 μM inhibitor, n = 4 for 1 μM inhibitor) or with 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium (n = 2 for both inhibitor concentrations) for 48 h prior to analysis of AsNOS expression. (C) Anti-phospho-INR Western blot of ASE cells stimulated with 1.7 μM human insulin or with 15.6 P. falciparum merozoites per cell for 5–30 min. Unstimulated ASE cells (NS) were used as controls (incomplete medium- and HEPES buffer-stimulated cells yielded identical results, not shown). Anti-phospho-INR antisera did not cross-react with P. falciparum merozoites (not shown). The figure shown is representative of replicated Western blots from independent experiments.

FIG. 6. Plasmodium falciparum merozoites signal A. stephensi cells and AsNOS induction through non-INR PTK. (A) ASE cells were pretreated for 1 h with HNMPA-(AM)3, an inhibitor of INR activation, dissolved in dimethyl sulfoxide or with an equivalent volume of dimethyl sulfoxide as a mock pretreatment (0 μM). After pretreatment, cells were stimulated with 1.7 μM human insulin in HEPES buffer (n = 7 for 0.1 μM inhibitor, n = 4 for 1 μM inhibitor) or with 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium (n = 2 for both inhibitor concentrations) for 48 h prior to analysis of AsNOS expression. (B) Anti-phospho-INR Western blot of ASE cells stimulated with 1.7 μM human insulin or with 15.6 P. falciparum merozoites per cell for 5–30 min. Unstimulated ASE cells (NS) were used as controls (incomplete medium- and HEPES buffer-stimulated cells yielded identical results, not shown). Anti-phospho-INR antisera did not cross-react with P. falciparum merozoites (not shown). The figure shown is representative of replicated Western blots from independent experiments. (C) ASE cells were pretreated for 1 h with genistein, a PTK inhibitor, dissolved in dimethyl sulfoxide or with an equivalent volume of dimethyl sulfoxide as a mock pretreatment (0 μM). After pretreatment, cells were stimulated with 1.7 μM human insulin or with 15.6 P. falciparum merozoites as in A (n = 3 for 10^{-5} μM inhibitor, n = 2 for 10^{-4} μM inhibitor). For panels A and C, AsNOS expression levels were divided by mock pretreatment expression levels to show relative effects of the inhibitor on parasite induction of AsNOS expression. Values represent means ± SEs. Data (inhibitor versus mock pretreatment) were analyzed using the Student t test; P values are shown.

FIG. 7. Treatment with kinase inhibitors induces JNK/SAPK phosphorylation in ASE cells. ASE cells were treated with 100 μM LY294002 for 30 min, 100 μM genistein for 1 h, 100 μM HNMPA-(AM)3 for 1 h or 40 μM PD98059 for 30 min. After stimulation, cells were collected and lysed as described in the text and applied to anti-phospho-JNK/SAPK Western blots. Signal intensity of each band is shown. The figure shown is representative of replicated Western blots from independent experiments.

Relative intensity 6.2 4.2 22.2 4.7 1

1 versus 3); identical results were obtained at 15 min after stimulation (not shown). Further, PD98059 pretreatment reduced ERK phosphorylation in response to merozoites to an undetectable level (Fig. 8B, lane 1 versus 4). Because PD98059 also inhibited parasite induction of AsNOS (Fig. 8A), we infer that some level of ERK phosphorylation is required for parasite induction of AsNOS.

To determine whether DSOR1, the predicted upstream activator of A. stephensi ERK, was necessary for P. falciparum and insulin induction of AsNOS, we silenced DSOR1 with RNAi in MSQ43 cells prior to stimulation. In DSOR1 double-stranded RNA-transfected cells, DSOR1 mRNA levels were undetectable from 6 h to 5 d posttransfection (Fig. 8C). For AsNOS induction assays, MSQ43 cells were stimulated at 18 h posttransfection with 15.6 merozoites per cell, 1.7 μM human insulin, medium or HEPES buffer for 48 h. DSOR1 RNAi reduced merozoite induction of AsNOS by 87% relative to mock transfected cells (P = 0.04), while insulin induction was reduced by only 41% relative to mock transfected cells (P = 0.007; Fig. 8D). These data mirrored levels of AsNOS induction observed following pretreatment with PD98059 (Fig. 8A) and confirmed that DSOR1 and its likely impact on ERK activation are necessary for P. falciparum induction of AsNOS.

Plasmodium falciparum merozoites require Akt/PKB for AsNOS induction. To determine whether P. falciparum signals AsNOS induction through activation of A. stephensi PI3-K and Akt/PKB, ASE cells were pretreated with LY294002 and wortmannin, PI3-K inhibitors, prior to stimulation with human insulin or P. falciparum merozoites. Pretreatment of ASE cells with 1 μM or 20 μM LY294002 had no effect on the insulin-mediated induction of AsNOS compared to controls (Fig. 9A). Similar results were obtained when cells were pretreated with 1 × 10^{-4} to 10 μM wortmannin prior to insulin stimulation (not shown). In contrast to our results, 10^{-2} to 100 μM LY294002 reduced insulin-stimulated steroidogenesis in A. aegypti ovary cells by 60% (46), suggesting that insulin signaling varies among mosquito cell types. Treatment with 1 μM or 20 μM LY294002 reduced AsNOS expression by 65% (P = 0.0001) and 51% (P = 0.0002), respectively, in merozoite-stimulated cells relative to controls (Fig. 9A), suggesting that PI3-K activity is critical to parasite induction of AsNOS. To verify this conclusion, we examined activation of Akt/PKB by Western blotting in ASE cells stimulated by human insulin and merozoites, then used RNAi-dependent gene silencing to de-
FIG. 8. Plasmodium falciparum merozoites activate ERK and require DSOR1 for AsNOS induction. (A) ASE cells were pretreated for 30 min with 0.04 μM or 4 μM PD98059, a MEK inhibitor, dissolved in dimethyl sulfoxide or with an equivalent volume of dimethyl sulfoxide as a mock pretreatment (0 μM). After pretreatment, cells were stimulated with 1.7 μM human insulin in HEPES buffer \((n = 8 \text{ for } 0.04 \text{ μM inhibitor, } n = 5 \text{ for } 4 \text{ μM inhibitor})\) or with 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium \((n = 2 \text{ for } 0.04 \text{ μM inhibitor, } n = 3 \text{ for } 4 \text{ μM inhibitor})\) for 48 h. AsNOS expression levels were divided by mock pretreatment expression levels to show relative effects of the inhibitor on insulin (line) or parasite (bars) induction of AsNOS. Values represent means ± SEs. Data (inhibitor versus mock pretreatment) were analyzed using the Student \(t\) test; \(P\) values are shown. (B) Anti-phospho-ERK Western blot of ASE cells pretreated with 0.4 μM PD98059 or dimethyl sulfoxide then stimulated with 1.7 μM human insulin or with 15.6 P. falciparum merozoites per cell for 5 min. Unstimulated ASE cells were used as controls (incomplete medium- and HEPES buffer-stimulated cells yielded identical results, not shown). Anti-phospho-ERK antisera did not cross-react with P. falciparum merozoites (not shown). The figure shown is representative of replicated Western blots from independent experiments. (C) RT-PCR signals the A. stephensi midgut epithelium through activation of Akt/PKB and ERK. Based on our results that P. falciparum merozoites and PfGPIs are not insulin-mimetic to A. stephensi cells. Based on observations that PfGPIs are insulin-mimetic to mammalian cells (7, 52), we hypothesized that merozoites and PfGPIs may be perceived as insulin-mimetic to A. stephensi cells. Because insulin induces lactate release rather than glucose uptake in D. melanogaster Kc cells (8), we assayed lactate release in ASE cells stimulated with 0.17, 1.7, or 17 μM human insulin or HEPES buffer from 1 to 8 h after treatment. At 4 h after treatment with 1.7 μM insulin, lactate release relative to control cells was maximal and nearly identical to the 1.3-fold induction reported for Kc cells (not shown) (8). Although these assays confirmed that A. stephensi cells were similar to Kc cells in response to insulin, stimulation with PfGPIs and merozoites failed to induce significant lactate release relative to control treatments (Fig. 10). Our data indicate that, while merozoites and PfGPIs activate mosquito cells through signaling proteins that are associated with insulin signal transduction pathways, neither merozoites nor PfGPIs are insulin-mimetic to A. stephensi cells.

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falciparum signals AsNOS induction in vitro through DSOR1/ERK- and PI3-K/Akt-dependent pathways, we reasoned that PfGPI signaling in vivo would recapitulate these pathway associations. To address this question, two separate cohorts of female A. stephensi were provided with a bloodmeal supplemented with 2.5 μM PfGPIs in 80% ethanol, a bloodmeal supplemented with an equivalent volume of 80% ethanol, or an unmodified bloodmeal. Midguts were dissected at 0 h (immediately after) and at 0.5 h after feeding and prepared for Western blots of ERK and Akt/PKB phosphorylation. At both time points, PfGPIs induced phosphorylation of ERK and Akt/PKB in the A. stephensi midgut epithelium relative to both controls (Fig. 11) indicating that merozoites (Fig. 8B and 9B) and PfGPIs signal through activation of A. stephensi Akt/PKB and ERK.

DISCUSSION

Current efforts to control malaria parasite transmission include the development of genetically modified Anopheles mosquitoes that exhibit enhanced refractoriness to Plasmodium spp. The search for antiparasite effectors has identified some promising targets in the immune-responsive mosquito, including melanotic encapsulation and the synthesis of toxic reactive oxygen species, antimicrobial peptides, and NOS, as well as putative nonself recognition factors and signaling cascades implicated in transducing responses to bacterial challenge (re-

FIG. 9. Plasmodium falciparum merozoites activate Akt and require Akt for AsNOS induction. (A) ASE cells were pretreated for 1 h with 1 μM or 20 μM LY294002, a PI3-K inhibitor, dissolved in 100% ethanol or with an equivalent volume of ethanol as a mock pretreatment (0 μM). After pretreatment, cells were stimulated with 1.7 μM human insulin in HEPES buffer (n = 2 for both concentrations of inhibitors) or with 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium (n = 3 for both concentrations of inhibitors) for 48 h. AsNOS expression levels were divided by mock pretreatment expression levels to show relative effects of the inhibitor on insulin (line) or parasite (bars) induction of AsNOS. Values represent means ± SEs. Data (inhibitor versus mock pretreatment) were analyzed using the Student t test; P values are shown. (B) Anti-phospho-Akt/PKB Western blot of ASE cells stimulated with 1.7 μM human insulin in HEPES buffer, 15.6 P. falciparum merozoites per cell in incomplete RPMI 1640 medium, or 1.7 μM human insulin in HEPES buffer and stimulated with 15.6 P. falciparum merozoites per cell, for 10 min or 30 min. Unstimulated ASE cells were used as controls (incomplete medium- and HEPES buffer-stimulated cells yielded identical results, not shown). Anti-phospho-Akt/PKB antisera did not cross-react with P. falciparum merozoites (not shown). Arrow indicates slower migrating, fully phosphorylated putative A. stephensi Akt/PKB. The figure shown is representative of replicated Western blots from independent experiments. C, RT-PCR of Akt expression levels following RNAi of A. stephensi Akt in mock control (C) and double-stranded RNA-transfected (T) MSQ43 cells from 6 h to 5 d posttransfection. Ribosomal S7 protein gene RT-PCR amplification was used as a loading control and to confirm sample integrity. (D) AsNOS expression levels in mock- and Akt double-stranded RNA-transfected cells were divided by expression levels in matched transfected cells stimulated with incomplete RPMI 1640 medium to show relative AsNOS induction by treatment with 15.6 P. falciparum merozoites per cell (n = 2), respectively. Values represent means ± SEs. Data within each treatment were analyzed using the Student t test; P values are shown.

FIG. 10. Plasmodium falciparum merozoites and PfGPIs do not mimic the effects of insulin on lactate release by A. stephensi cells. ASE cells were stimulated with 2.5 μM PfGPI in 80% ethanol (n = 2), with 15.6 P. falciparum merozoites per cell in incomplete medium (n = 2), or with 1.7 μM human insulin in HEPES buffer (n = 2) for 4 h. Matched control cells for each replicate were stimulated with equivalent volumes of HEPES buffer, incomplete medium or 80% ethanol. Lactate levels are represented as means ± SEs. Data within each treatment were analyzed using the Student t test.
viewed in reference 17). Although the repertoire of reported responses to malaria parasite infection and invasion may be confounded by resident microbial flora in Anopheles (56), significant expression of immune genes in antibiotic-treated, parasite-infected mosquitoes has consistently predicted the existence of Plasmodium-specific mechanisms of gene induction (34, 44). However, the identity of parasite-derived signaling factors capable of inducing specific responses in Anopheles cells has remained unknown until completion of the work described here.

In mammalian cells, PI(3)Ks are sufficient to account for the most notable effects of P. falciparum (53, 54, 57). We have demonstrated that 2.5 μM PI(3)Ks can induce ASNOS expression >5-fold in A. stephensi cells, results that are consistent with inductions of NO synthesis of 1.5-fold and 4-fold by 1 μM and 10 μM PI(3)Ks, respectively, in mouse macrophages (57). During parasite infection, induction of ASNOS expression in the midgut is biphasic, with >2-fold inductions at 6 h, 36 h, and 48 h after feeding (31). Provision of PI(3)Ks in the bloodmeal also induced a biphasic pattern of ASNOS expression in the mosquito midgut (Fig. 2C), with the earlier initial induction compared to natural infection (0 to 1 h versus 6 h) likely due to the more immediate availability of a larger concentration of PI(3)Ks in the midgut after feeding. The similarity of ASNOS induction patterns following feeding on PI(3)Ks and natural infection suggests that parasite PI(3)Ks are an important signal for ASNOS induction prior to (~24 h) and during parasite invasion (24 to 48 h) of the midgut.

Plasmodium falciparum and PI(3)Ks signal A. stephensi cells through insulin-responsive PI3-K/Akt and DSOR1/ERK. In mouse macrophages, PI(3)Ks induced rapid phosphorylation of ERK2, although PD98059 pretreatment suggested that ERK signaling was not involved in induction of macrophage NO synthesis by PI(3)Ks (63). In our studies with A. stephensi, stimulation of different target cells (ASE cells in vitro and midgut cells in vivo) with both whole parasites and PI(3)Ks revealed important information about ERK signaling. Although some level of ERK phosphorylation is necessary for ASNOS induction by P. falciparum in vitro (Fig. 8A and B), ERK phosphorylation by PI(3)Ks in vivo (Fig. 11) was more prominent.

In mammalian cells, nonphosphorylated, monophosphorylated, and fully bisphosphorylated forms of ERK2 are detectable (9, 60), with the balance of these forms in different cell types maintained by the opposing action of MEK1 and multiple protein phosphatases. Based on these observations, it was proposed that diverse signals may be integrated at the phosphatase level, rather than the kinase level, to dictate the cellular responses to external stimuli (61). Indeed, the activity of monophosphorylated ERK2 was determined to be intermediate to that of unphosphorylated and fully active bisphosphorylated ERK2 (62). The reduction in ERK phosphorylation within 5 min of parasite stimulation, together with our knock-out results, suggests that an ERK pool with diminished levels of bisphosphorylated ERK and perhaps higher levels of functional, monophosphorylated ERK drives the MEK-dependent cellular response to P. falciparum in ASE cells. As with ERK activation, we noted subtle differences in Akt activation in A. stephensi cells in vitro and in vivo. Specifically, PI(3)K-activated Akt/PKB phosphorylation in midgut cells (Fig. 11) did not result in the protein mass shift observed following P. falciparum stimulation of ASE cells (Fig. 9B). While some of these differences may be attributable to physiological differences between ASE and midgut cells, they also suggest that multiple signals from whole parasites, including at least PI(3)Ks and perhaps hemozoin (27, 38, 55) and others, contribute to ASNOS induction.

In addition to mimicry of insulin, PI(3)Ks signal mammalian cells through protein kinase Ce, PTK p59 hck, and nuclear factor-kBc-rel (57, 59). Additional data indicate that Toll-like receptors are activated by malaria parasites (28), a signaling mechanism that is well established for GPIs of the parasitic protozoan Trypanosoma cruzi (2, 6). Although our data indicate involvement of kinases associated with insulin signaling in ASNOS induction by P. falciparum and PI(3)Ks, these agents are not insulin-mimetic to A. stephensi cells. We conclude that activation of A. stephensi Akt/PKB and DSOR1/ERK by P. falciparum merozoites and PI(3)Ks is likely due to activation of pathways that share signaling components with insulin signal transduction pathways. Akt/PKB, for example, phosphorylates more than 50 known mammalian substrates associated with cell growth, defense, survival, and metabolism (25), suggesting that malaria parasite activation of A. stephensi Akt/PKB perturbs multiple pathways and physiological processes in A. stephensi cells. Further, inhibition of parasite signaling of ASNOS induction by genistein, a PTK inhibitor that is inactive against the INR (1), indicates that other PTKs, perhaps including representatives of the src family, are involved in parasite signaling of ASNOS induction. Therefore, mimicry of insulin by PI(3)Ks appears to be restricted to mammalian hosts of P. falciparum, but the conservation of PI(3)Ks as a prominent parasite-derived signal of innate immunity can now be extended to include Anopheles mosquitoes. This novel finding significantly increases the likelihood of identifying additional signaling pathways and downstream effectors associated with mosquito resistance to parasite development.

In general, the context of parasite signaling of ASNOS induction in the mosquito midgut is likely to be complicated by simultaneous exposure of midgut cells to dynamic concentrations of parasite-derived factors, mosquito-derived factors and exogenous factors in mammalian blood ingested during feeding. The latter factors include human insulin, which can induce ASNOS expression after feeding, and human transforming
growth factor -β1, which alters AsNOS induction and parasite infection in *A. stephensi* (31). We are challenged, therefore, to determine whether these factor(s) synergize or interfere with other signals, endogenous and exogenous in the blood-filled midgut, to mediate anti-parasite immunity in the mosquito. Cross talk among pathways of interest to us is well known from mammalian systems. For example, transforming growth factor β1-mediated growth inhibition is dependent on activation of IRS proteins (26), while the effects of transforming growth factor -β1 are regulated by PI3-K/Akt at the level of direct interaction between Akt and Smad3 (12, 43). Hence, an understanding of the complexity of the signaling milieu in the mosquito, which exhibits remarkable conservation with that in the mammalian host, is necessary for the success of efforts to manipulate signaling factors, pathways or effector genes to enhance mosquito refractoriness.

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