Original Contribution

Nitric oxide metabolites induced in Anopheles stephensi control malaria parasite infection

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Abstract

Malaria parasite infection in anopheline mosquitoes is limited by inflammatory levels of nitric oxide metabolites. To assess the mechanisms of parasite stasis or toxicity, we investigated the biochemistry of these metabolites within the blood-filled mosquito midgut. Our data indicate that nitrates, but not nitrites, are elevated in the Plasmodium-infected midgut. Although levels of S-nitrosothiols do not change with infection, blood proteins are S-nitrosylated after ingestion by the mosquito. In addition, photolyzable nitric oxide, which can be attributed to metal nitrosyls, is elevated after infection and, based on the abundance of hemoglobin, likely includes heme iron nitrosyl. The persistence of oxyhemoglobin throughout blood digestion and changes in hemoglobin conformation in response to infection suggest that hemoglobin catalyzes the synthesis of nitric oxide metabolites in a reducing environment. Provision of urate, a potent reductant and scavenger of oxidants and nitrating agents, as a dietary supplement to mosquitoes increased parasite infection levels relative to allantoin-fed controls, suggesting that nitrosative and/or oxidative stresses negatively impact developing parasites. Collectively, our results reveal a unique role for nitric oxide in an oxyhemoglobin-rich environment. In contrast to facilitating oxygen delivery by hemoglobin in the mammalian vasculature, nitric oxide synthesis in the blood-filled mosquito midgut drives the formation of toxic metabolites that limit parasite development.

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The mosquito Anopheles stephensi, a primary vector of human malaria in India and the Middle East, possesses a single copy of an *NO synthase gene (AsNOS) that is inducible by malaria parasite (Plasmodium spp.) infection [1,2]. Parasite development in the mosquito begins with the ingestion of blood containing sexual-stage gametocytes. Mobile zygotes or ookinetes penetrate the midgut epithelium 24–36 h later and transform into vegetative oocysts under the basal lamina in the hemolymph-filled, open circulatory system of the mosquito. Oocysts grow and develop for 10–12 days and then release thousands of haploid sporozoites, which invade the salivary glands and are released into the saliva during subsequent blood feeding.

During malaria parasite infection, induction of AsNOS expression in the midgut is biphasic, with greater than twofold inductions at 6 and 36–48 h after feeding [2], times associated with parasite development in the blood mass before invasion and after ookinete invasion of the midgut epithelium, respectively. Induction of NOS expression has been correlated with ookinete invasion as revealed by diaphorase staining [1] and immunofluorescence [3]. Synthesis of *NO limits parasite development in the mosquito midgut as shown by dietary provision of a NOS inhibitor (l-NAME) in the infectious blood meal [1]. The effect of midgut *NO synthesis on parasite

Abbreviations: AP, alkaline phosphatase; AsNOS, Anopheles stephensi nitric oxide synthase; GSNO, S-nitrosoglutathione; ICR, Institute of Cancer Research; metHb, deoxygenated hemoglobin; NAME, N⁶-nitro-l-arginine methyl ester; NO₂, nitrogen oxides; NTYR, nitrotyrosine; oxyHb, oxygenated hemoglobin; pBM, post-blood meal or post-blood feeding; PN, peroxynitrite; RNNOs, N-nitroso compounds; ROS, reactive oxygen species; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean; SNO, S-nitrosothiol.

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viability occurs before and during midgut invasion. In parallel studies, provision of L-NAME, but not D-NAME, to *A. stephensi* significantly decreased the percentage of apoptotic *Plasmodium berghei* ookinetes in the midgut lumen [4]. At later times during midgut invasion, ~5% of ookinetes travel five to six cell diameters in the epithelium, perhaps in an effort to escape the toxic diffusible 'NO and 'NO metabolites [3]. This extensive movement may explain the observation that ~95% of ookinetes seem to escape the epithelium [3] yet only a few remain to survive as oocysts [reviewed in [5]].

The direct and indirect effects of 'NO and 'NO metabolites on malaria parasite viability were previously known only from studies of mammal-dwelling asexual stage parasites. Minimal toxicity of free 'NO suggested that killing of *Plasmodium* occurs via the formation of nitrogen oxides (NO₃⁻) [6]. Specifically, Rockett et al. [6] found that low-molecular-weight 3-nitrosothiols (SNOs) displayed a 1000-fold greater toxicity to *Plasmodium falciparum* than nitrate, which was 3-fold more toxic than nitrite. In addition, at lower concentrations some NO₃⁻ were cytostatic to *P. falciparum* [7,8]. In contrast to our knowledge of asexual parasites, the effects of NO₃⁻ on mosquito-stage parasites are unknown.

In the present study, we have characterized the levels and types of NO₃⁻ produced in the mosquito midgut. In this context it is necessary to consider the nature of blood digestion within the mosquito midgut. During feeding, *A. stephensi* ingests 2–10 μl of blood that is concentrated to a volume of 1–2 μl by diuresis. The pH of the mosquito midgut is slightly less than 7 before the blood meal, but increases up to ~7 at 6–24 h post-blood meal (pBM) [9]. Net consumption of protein from blood is in the range of 550 μg, ~90% of which is Hb [10]. Over a period of 48 h, digestion occurs from the periphery of the blood bolus to the center. Intact erythrocytes are visible in the blood bolus center up to 24 h pBM. Free heme from Hb digestion is converted to hematin that adheres to the peritrophic membrane (PM), a polysaccharide matrix which surrounds the blood mass. At the completion of digestion, the remaining blood bolus and PM are excreted.

In previous work, we demonstrated that parasite-induced synthesis of 'NO generates an inflammatory state in the *A. stephensi* midgut [2]. Here we show that enhanced levels of nitric oxide metabolites are consistent with a toxic rather than a static effect on developing parasites. In addition, photolyzable 'NO, which can be attributed to metal nitrosyls, likely includes Hb-associated heme iron nitrosyl based on the molar abundance of Hb. The persistence of oxyHb throughout the course of blood digestion and changes in Hb conformation in response to parasite infection suggest that Hb may act to catalyze the synthesis of nitric oxide metabolites in a reducing environment.

**Experimental procedures**

**Insect rearing and parasite infection**

*A. stephensi* Liston (Indian wild type) mosquitoes were maintained on 10% sucrose at 27°C and 75% relative humidity under a 12-h light/dark cycle. Mosquitoes 4–7 days of age were blood fed on anesthetized naïve (uninfected) or *P. berghei*-infected (5–14% parasitemia; strain NK65) Institute of Cancer Research (ICR) mice. After the blood meal, mosquitoes were maintained at 21°C for parasite development. For feeding studies, 1 mM urate and 1 mM allantoin were dissolved in phosphate-buffered saline (PBS; 1.06 mM KH₂PO₄, 0.15 M NaCl, 5.6 mM Na₂HPO₄) and the pH was adjusted to 6.7. The NOS inhibitor L-NAME (1 mg/ml) or the inactive stereoisomer D-NAME (1 mg/ml) was provided as dietary supplement to the mosquitoes as described [1]. Fresh NAME solutions were prepared daily in sterile distilled, deionized water (ddH₂O); ddH₂O was used for controls. Sterile cotton balls soaked with dietary treatment solutions (changed twice daily) and sugar cubes (changed daily) were administered from 3 days before blood feeding until termination of the experiment. For urate and NAME feeds, *P. berghei*-infected mice were rotated among mosquito cartons (15 min per carton) to avoid mouse-to-mouse variability in parasite infection. Mosquito infections were monitored by counting oocysts on midguts stained with 0.1% mercuric chloride at 10 days postinfection.

**Chemiluminescence detection of 'NO**

Free and photolyzable 'NO was measured by photolysis–chemiluminescence detection [11]. Midguts (10 per sample) were dissected into 0.1 M sodium phosphate buffer (0.09 M Na₂HPO₄, 0.006 M NaH₂PO₄; pH 8) containing 100 μM DTPA or into 0.15 M NaCl (pH 7.4) containing 100 μM DTPA and injected into the manual injection valve of an Isco 2350 HPLC pump connected to a photolysis chamber (Nitrolite, Thermedics, Inc., Woburn, MA, USA). Degassed (with N₂ or He) ddH₂O was used as the mobile phase (1 ml/min flow rate). 'NO was released by photolysis when the sample passed through a quartz coil surrounding a mercury arc lamp emitting broad wave band UV light (Hanovia–Colight, Inc., Newark, NJ, USA). The output passed through two cold traps (0 and ~75°C) to condense the liquid and less volatile gasses (such as nitrate and nitrite). In the chemiluminescence spectrometer (Model 510, thermal energy analyzer; Thermedics, Inc.), decay of activated state 'NO₂⁻ was detected by a photomultiplier and the electrical output analyzed by an integrator (5890 Series II Plus, gas chromatograph; Hewlett Packard). Values obtained from the integrator were compared to a standard curve made from samples of known concentrations of 3-nitroso glutathione (GSNO). GSNO was prepared immediately before use by reacting equimolar concentrations (0.5 M) of glutathione in water with 100 μM DTPA and nitrite in 1 M HCl with 100 μM DTPA. Concentrations of midgut-bound 'NO were adjusted to heme concentration in each sample. All buffers were analyzed for NO₃⁻; detectable levels were subtracted from the sample data.

For nitrite and SNO analyses, chemical reductions were used to liberate 'NO before chemiluminescence detection. Mosquito midguts (50 per sample) were dissected into PBS with 1% Nonidet P-40, 4 mM K₂Fe(CN)₆, 10 mM NEM, and 0.1 mM DTPA, pH 7.4 [12]. Before analysis, midgut lysate sample proteins were concentrated by microfiltration (10 or 100 kDa Microcon; Millipore Corp., Bedford, MA, USA) at ~2000 g for
20 min; this treatment was essential to minimize sample foaming during analysis. In addition, 100 μl diluted antifoam agent (166 μl in 5 ml water; Sievers) was added to the reaction vessel to further reduce foaming.

For nitrite analyses, sample aliquots were injected into an anaerobic (He-purged) reaction vessel containing room temperature acidified KI (50 mg KI in 5 ml glacial acetic acid) for conversion of sample nitrates to NO [13]. For SNO analyses, samples were pretreated with HgCl₂ [1 part 0.2% HgCl₂ in water (or water alone for controls)] and rehydration through graded alcohols, the sections were rinsed with buffer S (0.5 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 4% formalin) at 1, 3, and 6 days pHBM. Ten-micrometer sections of paraffin-embedded mosquitoes were measured with a Sievers NO analyzer (Sievers Instruments, Boulder, CO, USA). Sample values were compared to standard curves of sodium nitrite (NaNO₂) and normalized against heme concentrations of matched sample aliquots to account for differences in blood meal volumes among mosquitoes. Induction of nitrite and SNO levels in midguts of P. berghei-infected A. stephensi relative to those in uninfected A. stephensi at each time point were analyzed with Student’s t test.

**Immunohistochemical staining for nitrotyrosine (NTYR)**

Detection of NTYR was adapted from protocols of Crow and Ischiropoulos [14]. Non-blood-fed, P. berghei-infected and uninfected A. stephensi were collected and preserved in Lillie’s neutral buffered formalin (29 mM NaH₂PO₄, 1 part 0.2% HgCl₂ in water (or water alone for controls)); 2 parts mosquito lysate: 3 parts PBS) for 30 min at room temperature. Acidified KI was used to chemically reduce nitrates to SNOs to NO. Liberated NO from KI-treated and from HgCl₂/KI-treated samples was measured with a Sievers NO analyzer (Sievers Instruments, Boulder, CO, USA). Sample values were compared to standard curves of sodium nitrite (NaNO₂) and normalized against heme concentrations of matched sample aliquots to account for differences in blood meal volumes among mosquitoes. Induction of nitrite and SNO levels in midguts of P. berghei-infected A. stephensi relative to those in uninfected A. stephensi at each time point were analyzed with Student’s t test.

**Hemoglobin analysis**

At various times pHBM, mosquitoes were collected and midguts dissected into the appropriate buffer (see below; 9 μl per midgut). Midguts were homogenized by sonication and centrifuged at 2000 g for 2 min and the supernatant was collected and immediately stored at −80°C. Midgut lysate heme concentrations were calculated based on absorbance values at 300–800 nm; heme concentrations were calculated from the absorbance peaks and the respective extinction coefficients for either oxyHb (ε₅₅₀ = 125 M⁻¹ cm⁻¹, ε₅₄₁ = 14.6 M⁻¹ cm⁻¹, ε₆₈₀ = 13.8 M⁻¹ cm⁻¹) or methemoglobin (metHb; ε₅₅₀ = 124 M⁻¹ cm⁻¹).

A. stephensi midguts (10 per sample) were dissected into PBS at 1, 12.5, and 24 h pHBM from infected and uninfected blood-fed mosquitoes and from non-blood-fed mosquitoes. Mouse tail blood samples were collected from both infected and uninfected mice immediately before blood feeding and diluted 1:10 in PBS. Heme concentrations in midgut lysates and mouse blood samples were measured and samples containing 3 nmol heme in 5 μl PBS were mixed with 5 μl loading buffer (62.5 mM Tris–HCl, pH 6.8, 40% glycerol, with or without 2% β-mercaptoethanol). Proteins were electrophoretically separated on 7.5% polyacrylamide gels under native conditions. Hemoglobins were visible without staining as heme-containing red-brown bands. The electrophoretically separated proteins were transferred to Immobilon–P (Millipore) using wet transfer (EC140 Mini Blot Module; E-C Apparatus Corp.) and Towbin transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) as described by the manufacturer. Blots were treated with 1 μl levensomile (pH 7.5) for 20 min to block endogenous alkaline phosphatases (AP) and then blocked with 1 μl in TBS (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris base, pH 7.4) containing 5% nonfat dry milk powder and 0.02% Tween 20. For detection, Western blots were incubated with rabbit anti-mouse Hb antisera (Research Plus, Inc.) at a 1:100,000 dilution overnight at 4°C followed by AP-conjugated goat anti-rabbit IgG secondary antibody (Southern Biotechnology Associates) at 1:10,000 for 2 h at room temperature. The colorimetric 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrates (Vector) were used for detection.

**SNO–biotin-switch Western blotting**

SNO–biotin-switch Western blots were prepared using a protocol adapted from Jaffrey and Snyder [15]. Briefly, midguts from uninfected and P. berghei-infected mosquitoes collected at 1, 6, 12, and 24 h pHBM were dissection into buffer A (69 mg NEM, 4.3 mg DTPA, 49 mg potassium acetate in 10 ml H₂O₂, pH 6.5). Immediately before blood feeding, samples of mouse tail blood were obtained and diluted 1:10 into buffer A. Heme concentrations of midgut lysates and mouse blood samples were measured and samples containing 1.5 nmol heme in 3 μl buffer were mixed with equal volumes of 50 μM NEM containing 2.5% SDS and incubated for 30 min at 37°C. An equal volume of 1 mM CuCl₂ was added to each sample; samples were then incubated for 30 min at 37°C. Subsequently, 0.5 vol of 4 mM...
bion–HPDP (N-[6–(bionamido)hexyl]-3’-(2’-pyridyldithio) propionamide; US Alchemy, Inc.) in DMSO was added to each sample; samples were then incubated for 30 min at room temperature. For controls, a duplicate sample set was processed identically except that DMSO without biotin–HPDP was added in the last step. All reactions were performed in the dark. Sample proteins were mixed without heating with nonreducing Laemmli’s sample buffer, electrophoretically separated on 10–20% Tris–HCl SDS–PAGE Ready gels (Bio-Rad), and then transferred to Immobilon-P (Millipore) using semidy transfer. Membranes were blocked with 10% milk before incubation with a 1:30,000 dilution of peroxidase-conjugated anti-biotin monoclonal antisera (Clone BN-34; Sigma). Peroxidase activity was detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

Statistics

Statistical significance was evaluated using Student’s t test (Microsoft Excel).

Results

Nitrites and SNOs in the mosquito midgut are not increased by parasite infection

We previously reported levels of total °NO metabolites in midgut lysates from P. berghei–infected and uninfected A. stephensi [2]. These data were derived from samples analyzed utilizing vanadium chloride reduction and thus reflect levels of total nitrogen oxides. At 12.5 h pBM, total NO_4 levels were 5.9±1.0 μmol/mmol heme in P. berghei-infected A. stephensi and 4.0±0.6 μmol/mmol heme in uninfected A. stephensi [2]. At 24 h pBM, these levels were 13.2±3.9 μmol/mmol heme in infected and 8.7±1.9 μmol/mmol heme in uninfected A. stephensi [2]. During blood digestion, heme concentrations decreased predictably over time due to blood digestion but did not differ between midgut lysates from parasite-infected and those from uninfected mosquitoes within any time point (Fig. 1A). At 12.5 h, the average midgut lysate heme concentration was ~17 mM and at 24 h, the average heme concentration was ~15 mM (Fig. 1A). Therefore, one can conclude that the increases in total °NO metabolites resulted from increased °NO production [2] rather than infection-induced changes in digestion. However, the chemical nature of the °NO metabolite(s) within the midgut remained unclear. Therefore we examined the proportion of these metabolites existing as nitrites and SNOs (Fig. 1B). The proportions of nitrites and SNOs to total °NO metabolites in uninfected and infected midgut lysates were not significantly different at 12.5 and 24 h after feeding and, in fact, followed a trend of lower proportions in infected lysates relative to uninfected lysates at 12.5 h (Fig. 1B). These observations indicated that enhanced levels of total °NO metabolites in infected midguts relative to uninfected midguts were due to significantly enhanced levels of nitrites and higher NO_3.

In addition to a lack of differences in proportions at 12.5 and 24 h after infection, absolute levels of nitrites did not increase in response to parasite infection. Specifically, at 12.5 h pBM, midgut lysate nitrite levels were 1.6±0.3 μmol/mmol heme in P. berghei-infected mosquitoes and 2.2±0.4 μmol/mmol heme in uninfected mosquitoes. At 24 h pBM, midgut lysate nitrite levels were 3.0±0.5 μmol/mmol heme in P. berghei-infected mosquitoes and 3.1±0.8 μmol/mmol heme in uninfected mosquitoes.

As observed for nitrites, absolute levels of SNOs did not increase significantly in response to parasite infection. At 12.5 h pBM, midgut lysate SNO levels were 2.9±1.1 μmol/mmol heme in P. berghei-infected mosquitoes and 2.6±0.9 μmol/mmol heme in uninfected mosquitoes. At 24 h pBM, midgut lysate SNO levels were 3.9±1.7 μmol/mmol heme in P.
bergheri-infected mosquitoes and 2.9±1.0 μmol/mmol heme in uninfected mosquitoes.

Tyrosine nitration is enhanced by parasite infection

The prior results suggested that chemistry of higher oxides of nitrogen might prevail within the mosquito midgut. As a marker of such chemistry [16], we selected NTYR. Much greater NTYR levels were observed in tissues of P. bergheri-infected versus uninfected A. stephensi at 24 h pBM (Fig. 2), suggesting that parasite-induced higher oxides of nitrogen observed at this time postinfection ([2] and Fig. 1) were temporally associated with nitration. Staining was particularly evident in the fat body, ovarian tissue, midgut epithelium, and blood bolus (Fig. 2). Staining was also more intense in the distal portion of the midgut, consistent with observations that parasite invasion and oocyst development are concentrated in this area [reviewed in [17]]. Together, midgut NOx levels and NTYR data suggested that *NO-mediated stress is enhanced in and around the infected midgut during the time of ookinete invasion of the midgut epithelium and oocyst development.

Hemoglobin in the mosquito midgut is oxygenated throughout digestion

UV–visible spectra of midgut lysates from 1 to 49 h pBM (Fig. 3) were used to calculate midgut heme concentrations (Fig. 1). From spectral analyses, we noted that Hb within the mosquito midgut, regardless of time pBM or presence or absence of infection, seemed to be maintained in a ferrous liganded or oxyHb state without detectable heme oxidation to 33 h pBM (Fig. 3). The lack of oxidized heme in A. stephensi midgut lysates suggested that the midgut is a reducing environment [18]. As oxyHb reacts readily with *NO, we chose to examine whether *NO remained bound to Hb in the midgut by means of photolysis with chemiluminescence and Western blotting.

Photolyzable adducts of *NO in the mosquito midgut are increased by parasite infection

Photolysis coupled with chemiluminescence detection is a sensitive measure of SNOs and metal-nitrosyl adducts and to

Fig. 2. Nitrotyrosine (NTYR) levels in the midgut and surrounding tissues are increased in response to P. bergheri infection. Immunohistochemical staining of 10-μm sections of blood-fed A. stephensi was performed using polyclonal anti-nitrotyrosine. At 24 h pBM, increased staining for NTYR (purple) was observed in tissues of (A, B) P. bergheri-infected compared to (C, D) uninfected A. stephensi. Staining was observed in the (b) blood mass, (e) ovarian tissue, (f) fat body, and (→) midgut epithelium. The most pronounced staining occurred in the blood-meal bolus in the posterior midgut. Tissue samples were observed under 100× (A, C) and 400× (B, D) magnification; scale bar, 75 μm.

Fig. 3. Absorbance spectra of midgut hemoglobins. Spectra of lysates from parasite-infected and uninfected midguts collected from 1 to 33 h pBM were consistent with spectra for oxyHb and contained little to no detectable metHb. Representative spectra from 12.5 h pBM are shown here.
some extent N-nitroso compounds [19]. Photolyzed *NO levels in midgut lysates were measured immediately after (0 h) to 33.5 h after *A. stephensi* feeding on uninfected or *P. berghei*-infected mice. In all samples, concentrations of photolyzed *NO were 3.5-to 17-fold lower than corresponding concentrations of nitrates and SNOs in both parasite-infected and uninfected *A. stephensi* midgut lysates, indicating a lack of background detection of SNOs generated spuriously by reactions of abundant blood-derived thiols with nitrate. Photolyzed *NO levels of infected and uninfected midgut lysates were not significantly different at collection times <10 h (Fig. 4A). However, at 12.5–13.5 and 24–25.5 h pBM, the concentrations of photolyzed *NO* were significantly higher in infected compared to uninfected midgut lysate samples (Fig. 4B).

**Fig. 4. Midgut levels of photolyzed *NO* were increased in response to *P. berghei*-infection. Photolyzable *NO* was quantified using photolysis–chemiluminescence spectroscopy. For midgut lysates, *NO* concentration was normalized against sample heme concentration to account for variation in ingested blood meal volume.** (A) A representative timed series demonstrates that from immediately after (0 h) to 8 h pBM, photolyzed *NO* levels in uninfected and *P. berghei*-infected midguts were not significantly different. In contrast, elevated photolyzed *NO* levels were detected in infected midguts between 12.5 and 30 h pBM. (B) Parasite infection increased levels of photolyzed *NO* in midgut lysates collected from 8 to 33.5 h pBM (n=3). Values are represented as the concentrations of photolyzed *U NO* from immediately after (0 h) to 8 h pBM, photolyzed ingested blood meal volume. (A) A representative timed series demonstrates that normalized against sample heme concentration to account for variation in *P. berghei* and *A. stephensi* midguts contain SNO proteins and cross-linked Hb

Based on the detection of significant levels of SNOs within the blood-filled *A. stephensi* midgut we chose to examine the formation of SNO proteins as a function of time postfeeding because the nature of SNO-modified peptides and proteins could influence the toxicity of *NO* in the midgut [6,11]. To assess the profile of S-nitrosylated midgut proteins in *A. stephensi*, lysates were assayed using the “SNO–biotin-switch” method [15]. Western blots of heme-normalized midgut lysate samples from *P. berghei*-infected and uninfected *A. stephensi* showed little to no differences in the profiles of SNO-modified proteins at 1, 6, 12, and 24 h after blood feeding in two independent trials (lanes 3 and 4, Fig. 5A). Equivalent concentrations of heme-normalized mouse blood samples, drawn from mice before blood feeding, were used as controls (lanes 1 and 2, Fig. 5A). SNO-modified mouse proteins were detected and no obvious differences were noted between infected and uninfected blood, consistent with the fact that ICR mice do not produce *NO* in response to parasite infection [20]. Although SNO–protein profiles in the mosquito midgut did not change with parasite infection, SNO proteins in mouse blood differed from SNO proteins in blood-fed mosquito midguts at all times examined (lane 1 versus 3 and lane 2 versus 4 for each blot, Fig. 5A). These observations suggested that exposure of blood to the mosquito midgut alters SNO-protein content and that production of *NO* by the mosquito midgut leads to S-nitrosylation of proteins within the blood meal.

As expected, digestion of midgut blood over time seemed to reduce protein complexity in the samples (lanes 3 and 4 at 24 h versus at 1 h, Fig. 5A). A strong cross-reacting band at ~64 kDa, the molecular mass of tetrameric Hb, was evident in the SNO–biotin-switch blots at 12 h (T2, lanes 3 and 4) and particularly at 24 h in both mosquito lysates and mouse blood samples (lanes 1–4, Fig. 5A). It is probable that this protein is tetrameric Hb, as this is the predominant protein at that molecular weight within these samples (Fig. 5B).

To further examine the structure of Hb in midgut lysates from parasite-infected and uninfected *A. stephensi*, lysate proteins were separated electrophoretically under native conditions. Hemoglobin can be directly observed under these conditions without the use of staining dyes due to the red-brown color derived from heme. Tetrameric Hb was evident as the most abundant band in normal mouse blood and in most midgut lysates (Figs. 6A and 6B). However, in midgut lysates from parasite-infected *A. stephensi* collected 12.5 and 24 h pBM, tetrameric Hb was faint or absent, whereas a much more slowly migrating red-brown protein was abundant (arrows, Fig. 6A). This slowly migrating protein was not present in normal mouse blood, uninfected midgut lysates, or samples collected at 1 h pBM (Fig. 6A). When samples were pretreated before electrophoresis with β-mercaptoethanol, the slowly migrating protein disappeared and a protein with migration identical to that of tetrameric Hb appeared in midgut lysates from 12.5-and 24-h parasite-infected mosquitoes (Fig. 6B). As expected, pretreatment with β-mercaptoethanol had no effect on tetrameric Hb in

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the remaining samples (Fig. 6B). These results suggested that disulfide bonds were responsible for retarding the migration of an aggregated Hb.

To confirm that the slowly migrating protein was Hb, anti-Hb Western blots were performed on midgut lysates collected at 24 h pBM and separated and transferred under native conditions. As expected, the red-brown proteins in midgut lysates from both uninfected and infected \textit{A. stephensi} were clearly identifiable as Hb (Fig. 6C). The association of this slowly migrating Hb with only infected midguts at later times pBM suggested that nitrosative or oxidative stress may be associated with its formation. Interestingly, a minority of infected midgut lysates lacked the slowly migrating Hb (not shown), suggesting that variation in parasite infection intensity and, hence, nitrosative stress can be translated into variation in disulfide-bonded Hb formation.

Dietary supplementation with urate, but not allantoin, increased parasite infection levels in \textit{A. stephensi}

The observation of increased NTYR staining in the mosquito midgut demonstrated that parasite infection leads to an increase in both nitrosative and oxidative stress. Uric acid/urate is a product of purine metabolism with limited membrane

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**Fig. 5.** Parasite infection does not alter the profile of SNO-modified proteins, which may include Hb, in the \textit{A. stephensi} midgut. (A) SNO–biotin-switch Westerns revealed S-nitrosated protein profiles in infected versus uninfected midgut lysates collected from 1 to 24 h pBM. Although uninfected and infected midgut lysate profiles were not different (lane 3 versus 4 in each blot), SNO-modified proteins in mouse blood were altered after ingestion by \textit{A. stephensi} (lane 1 versus 3 and lane 2 versus 4 for each blot). (B) A Coomassie-stained gel (a) and Western blot (b) using anti-Hb antisera for 12-h-pBM T2 midgut lysate samples (from A). Migration of tetrameric (t), dimeric (d), and monomeric (m) Hb is indicated by arrows. Lanes: 1, uninfected mouse blood; 2, \textit{P. berghei}-infected mouse blood; 3, uninfected midgut lysate; 4, infected midgut lysate; and 5, molecular weight markers (kDa). T1 and T2 refer to two independent trials.

**Fig. 6.** Midguts from parasite-infected mosquitoes contain a disulfide-linked, slowly migrating Hb at 12.5 and 24 h pBM. Uninfected mouse blood (M) and midgut lysates from non-blood-fed \textit{A. stephensi} (N) and \textit{P. berghei}-infected (I) and uninfected (U) \textit{A. stephensi} collected at 1, 12.5, and 24 h pBM were electrophoretically separated through 7.5% polyacrylamide. The heme-containing Hb’s were visible as red-brown bands on unstained gels. (A) Under native conditions, a slowly migrating Hb (arrows), relative to the expected tetrameric Hb (t), was visible in infected midgut lysates only at 12.5 and 24 h pBM. (B) Sample pretreatment with \(\beta\)-mercaptoethanol eliminated the slowly migrating Hb (arrows) and was coincident with the reappearance of tetrameric Hb (t), indicating that altered protein migration was due to disulfide bonds. (C) Western blot analysis using mouse anti-Hb antisera confirmed that the slowly migrating protein was Hb (arrow).
permeability and is a potent reductant and nonspecific scavenger of nitroxyl [21], PN [22], and ‘OH [23]. In other systems, PN and nitroxyl can induce NTYR formation, although nitroxyl does so only weakly [24]. In mice, urate administration to animals with symptoms of multiple sclerosis significantly decreased NTYR formation in brain tissue and greatly reduced disease severity relative to control animals [22].

We examined the effects of administration of urate, or its non-redox-active metabolite allantoin, by dietary supplementation to adult female mosquitoes. In preliminary assays, mosquito resting and flight behavior, mortality, and blood-feeding success among the urate-, allantoin-, and PBS-treated control groups were not markedly different. As such, we concluded that urate and allantoin were not overtly toxic to treated mosquitoes. The mean numbers of *P. berghei* oocysts from mosquitoes fed PBS and those fed 1 mM allantoin were not significantly different from each other (Fig. 7A). However, mean numbers of oocysts in both PBS and allantoin control groups were significantly lower than the mean number of oocysts in *A. stephensi* fed 1 mM urate (Fig. 7A). Human plasma contains urate at concentrations up to 500 μM [25], whereas mouse plasma contains ~20 μM urate [22]. If *A. stephensi* could utilize urate ingested with blood as a reductant and antioxidant, significant differences in infection could result from feeds that utilize human versus mouse blood. To test this possibility, a follow-up assay included dietary supplementation of urate at 500 μM and 1 mM. As observed previously, the mean number of oocysts in mosquitoes fed 1 mM urate was significantly higher than the mean number of oocysts in mosquitoes fed PBS (Fig. 7B). However, the number of oocysts from mosquitoes fed 500 μM urate was not significantly different from that of the PBS-fed controls (Fig. 7B).

**Discussion**

Previously we have shown that ‘NO synthesis limits *Plasmodium* development in *A. stephensi*. In the present study we have investigated the nature of the ‘NO metabolites involved in this form of insect immunity. During parasite infection, the lower oxides of nitrogen, namely nitrates and SNOs, were not increased relative to levels in uninfected insects, suggesting that these metabolites either are not involved in the anti-parasite response or form a more labile pool that was not detected in our assays. Further, given that autoxidation of ‘NO leads to a 50:50 ratio of nitrates and nitrites, the skewed production of nitrates indicates that there is an elevated production of higher oxides of nitrogen. Parasite infection in *A. stephensi* also resulted in increased levels of NTYR and photolyzable ‘NO adducts. Formation of these ‘NO metabolites in response to parasite infection seems to involve Hb, which is structurally modified in infected insects, yet maintained in a liganded form. The importance of oxidative and/or nitrosative stress to the control of parasite development is emphasized by the ability of dietary urate relative to control diet allantoin to increase parasite loads.

Free ‘NO and ‘NO adducts, including SNOs, N-nitroso compounds (RNNOs), and metal nitrosyls can contribute to signal output of photolysis with chemiluminescence detection [26]. Under conditions found in the *A. stephensi* midgut, it is likely that free ‘NO reacts rapidly to generate a variety of ‘NO metabolites. Although RNNOs may be generated endogenously [27], it is unlikely that they are involved in host defense as they do not possess antiplasmodial activity [28]. Because the steady-state concentration of SNOs was not elevated after infection, metal nitrosyls would seem to account for the increase in photolyzable ‘NO. Due to the high concentration of Hb within the midgut, it is reasonable to propose that the predominant metal nitrosyl is iron nitrosylHb. Alternatively, iron released from Hb during digestion before sequestration in hematin could react with ‘NO to form dinitrosyl iron complexes (DNICs) [29]. DNICs, although perhaps formed at lower levels than iron nitrosylHb, could mediate toxic effects through redox destabilization [30].

**Possible mechanisms for iron nitrosylHb formation in the mosquito midgut**

Classically, ‘NO can react with oxyHb to form metHb and nitrate or with deoxyHb to form iron nitrosylHb [31]. The levels of nitrosylHb in mammalian circulation have been reported to be ~5 μM in mixed venous blood and ~2.5 μM in arterial blood [32]. When mosquitoes feed, blood is drawn from the peripheral venous circulation, thus the blood meal likely contains nitrosylHb. However, the observation that midgut

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**Fig. 7.** Dietary supplementation with urate, but not allantoin, increased parasite infection levels in *A. stephensi*. Values are represented as means±SEM. (A) Dietary urate (1 mM) in PBS, but not allantoin (1 mM) in PBS, or PBS alone, increased *P. berghei* infection levels in *A. stephensi* as measured by 10-day oocyst count (p=0.006 for PBS versus urate, p=0.296 for PBS versus allantoin, and p=0.008 for urate versus allantoin; n=29). (B) Dietary urate increased parasite infection levels when provided at 1 mM but not at 0.5 mM relative to control PBS (0 mM) treatment (p=0.04 for PBS versus 1 mM urate, p=0.33 for PBS versus 0.5 mM urate, and p=0.03 for 0.5 mM versus 1 mM urate; n=20).
blood contains and maintains oxyHb suggests that ingestion of the blood meal is analogous to blood entering the lungs. The introduction of oxygen to partially nitrosylated Hb leads to a loss of the NO moiety through oxidation or conversion to SNO. However, under alkaline oxygenated conditions such as those found in the midguts of *Anopheles* spp. [9], iron nitrosylHb is reasonably stable and undergoes reversible oxygen binding with moderate affinity [33].

The addition of superoxide dismutase (SOD) to Hb increases the yield of total *NO* bound to Hb [34]. SOD activity is increased by blood feeding in *A. gambiae* by 0.95-to 1.94-fold at 12 h pBM and 2.1-to 2.5-fold at 24 h pBM relative to unfed controls [35], suggesting that mosquito SOD could facilitate *NO* binding to Hb. Finally, SOD can decrease the conversion of oxyHb to metHb [36], which would be consistent with the lack of observed metHb in blood-fed midguts.

All reactions of *NO* with Hb in the vasculature are decreased by the red blood cell (RBC) membrane [37], as *NO* reacts at least 1000 times faster with free Hb than with Hb contained by the RBC membrane [38]. The midgut conditions resemble that of a cell-free Hb system because the RBCs nearest to the midgut epithelium, the source of *NO*, are actively digested and lysed. The higher concentrations of Hb in the midgut together with high local concentrations of *NO* produced by the midgut epithelium could support nitrosylHb formation as described by Gow et al. [34]. Further, the reaction of *NO* with oxyHb to form nitrosylHb may be favored in the parasite-infected midgut, where in addition to high local *NO* fluxes, Hb is freed from RBCs and sequestered by disulfide bond-dependent aggregation during digestion.

**NTYR formation in the *A. stephensi* midgut**

In addition to NOS, a putative dual-function oxidase, *Ag-Duox*, is highly expressed in the *A. gambiae* midgut in response to ookinete invasion [39]. The *Ag-Duox* gene encodes an N-terminal peroxidase domain and a C-terminal NADPH oxidase domain [39]. As such, the local induction of *Ag-Duox* could lead to high local levels of O$_2^-$ as has been shown in *A. albimanus* upon injection of ookinete into midgut explants [40]. Based on these observations, we predicted that higher oxides of nitrogen are formed in *Anopheles* mosquitoes and that these metabolites could induce NTYR formation. In this regard it is important to note that the combination of *NO* and O$_2^-$ has been show to kill 75% of *P. berghei* ookinetes in vitro [40]. Our data showed that NTYR levels were significantly higher in parasite-infected *A. stephensi* relative to blood-fed, uninfected mosquitoes at 24 h pBM. In parasite-infected *A. stephensi*, elevated NO$_x$ were detected in the blood meal at 12.5 and 24 h pBM [2], a period consistent with enhanced NTYR staining at 24 h and that spans the final stages of ookinete maturation in the midgut and the beginning of ookinete invasion of the midgut epithelium.

The presence of NTYR suggests PN-like reactivity and it is likely that PN or its redox congeners directly affect parasite development. Diffusion of *NO* into the blood mass and the subsequent formation of toxic NO$_x$ would occur in the same physical environment in which critical stages of parasite development occur. Whereas NO$_x$ in general would be predicted to damage several classes of parasite biomolecules, we can speculate that some parasite proteins may be key targets for inhibition. For example, *P. falciparum* glutathione reductase (PfGR) can be inactivated by PN through nitrification of Tyr 106 and Tyr 114 [41]. These findings have contributed to strategies for developing irreversible PfGR inhibitors for novel antimalarial drugs [41], suggesting that similar investigations of mosquito-stage parasites could lead to the development of novel transmission-blocking strategies.

In light of the induction of SOD expression in the *A. gambiae* midgut after blood feeding [35], it is necessary to consider reactions independent of PN that may contribute to NTYR formation. For example, nitroxy, heme proteins, transition metals, and/or peroxidases can catalyze NTYR formation. Intense NTYR staining in the blood bolus may, therefore, reflect heme iron catalysis of tyrosine nitrination in an environment of Hb digestion and high *NO* flux [42]. Kumar et al. [39] hypothesized that *NO* synthesis after parasite infection in *A. gambiae* results in accumulation of nitrite, which is a substrate for myeloperoxidase-mediated tyrosine nitration [43]. However, all of these reactive pathways rely on the formation of higher NO$_x$ and thus NTYR can be considered a marker of nitrative stress.

**Urate enhances parasite development by reducing nitrative and oxidative stress**

Provision of urate as a dietary supplement increased parasite loads in *A. stephensi* relative to allantoin–fed control insects. Uric acid inhibits nitrination reactions and has been used as a scavenger of PN [22]. In addition, urate can alter the chemistry of nitroxyl [21], which could indirectly influence PN formation. Urate can also scavenge *OH* [23], suggesting that urate supplementation could have increased parasite infection by reducing levels of cytotoxic *OH*. In contrast to the direct effects of urate on NO$_x$ and ROS, urate does not interfere with peroxidase-mediated nitrination. As such, the effects of urate supplementation suggest that PN rather than an alternative metabolite is responsible for the bulk of the tissue nitrination and limiting parasite development.

**Toxicity of NO$_x$ predicted to occur in the *A. stephensi* midgut**

In the redox-active mosquito midgut, *NO* synthesis in the context of blood digestion results in the formation of NO$_x$ that likely mediate the *NO*-dependent reduction in *Plasmodium* development. Specifically, we propose that iron nitrosylHb and perhaps DNICs formed during blood digestion preserve the bioactivity of *NO* in the blood-filled midgut, whereas toxic nitrates and higher oxides of nitrogen comprise the majority of *Plasmodium*-induced NO$_x$. Further, the patterns of SNO-modified proteins change over time in the mosquito midgut, indicating that mosquito physiology drives dynamic S-nitrosylation of ingested blood proteins and perhaps endogenous mosquito proteins. These changes occur during the first 24 h of parasite development, a critical time in which parasites undergo
rapid developmental changes within the blood mass. Importantly, "NO synthesis occurs in the context of ROS produced in the midgut after feeding, a situation which would potentiate nitrative stress. In addition to mosquito-generated ROS, the ingested blood itself may be a source of oxygen radicals. Within 20 min of feeding, erythrocyte hemolysis frees up to 10% of the total ingested hemoglobin in the midgut lumen [44]. The globins are dissociated from heme and digested, whereas the heme is polymerized to insoluble hematin under oxygenated, alkaline conditions [45]. Polymerization, however, is not instantaneous, nor does it completely abolish radical chemistry [46]. Thus, significant quantities of heme and hemein can react with iron and oxygen in the blood meal to form ROS that could react with "NO to induce nitration in the blood mass and in the surrounding tissues. Intriguingly, elevated levels of nitrosylHb and NTYR are detected during the inflammatory response of two mouse strains to the protozoan *Leishmania amazonensis* [47], revealing a parallel to the chemistry and toxic effects of "NO synthesis in *Plasmodium*-infected *A. stephensi*.

Nitroxyln and PN are strongly reactive and can oxidize thiols and amines, nitrosylate metals, induce DNA strand breaks and base oxidation, oxidize lipids and proteins, deaminate DNA, nitrosylate metals, induce DNA strand breaks and...
Yang, T.; Olsen, K. W. Enzymatic protection from autoxidation for S.


