Inducible Peroxidases Mediate Nitrination of *Anopheles* Midgut Cells Undergoing Apoptosis in Response to *Plasmodium* Invasion*

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Plasmodium berghei invasion of *Anopheles stephensi* midgut cells causes severe damage, induces expression of nitric-oxide synthase, and leads to apoptosis. The present study indicates that invasion results in tyrosine nitration, catalyzed as a two-step reaction in which nitric-oxide synthase induction is followed by increased peroxidase activity. Ookinete invasion induced localized expression of peroxidase enzymes, which catalyzed protein nitrination in *vivo* in the presence of nitrite and H₂O₂. Histochemical stainings revealed that when a parasite migrates laterally and invades more than one cell, the pattern of induced peroxidase activity is similar to that observed for tyrosine nitration. In *Anopheles gambiae*, ookinete invasion elicited similar responses; it induced expression of 5 of the 16 peroxidase genes predicted by the genome sequence and decreased mRNA levels of one of them. One of these inducible peroxidases has a C-terminal oxidase domain homologous to the catalytic moiety of phagocyte NADPH oxidase and could provide high local levels of superoxide anion (O₂⁻), that when dissimulated would generate the local increase in H₂O₂ required for nitrination. Chemically induced apoptosis of midgut cells also activated expression of four ookinete-induced peroxidase genes, suggesting their involvement in general apoptotic responses. The two-step nitrination reaction provides a mechanism to precisely localize and circumscribe the toxic products generated by defense reactions involving nitration. The present study furthers our understanding of the biochemistry of midgut defense reactions to parasite invasion and how these may influence the efficiency of malaria transmission by anopheline mosquitoes.

Anopheline mosquitoes are the natural vectors of human malaria worldwide. When a female mosquito takes a blood meal from a malaria-infected host, the ingested *Plasmodium* gametocytes complete their differentiation into mature gametes in the midgut lumen. Following fertilization, zygotes mature into motile ookinetes, which traverse the midgut epithelium and form oocysts in the space between the epithelial cells and the basal lamina. Oocysts grow, mature, and eventually rupture, releasing a large number of sporozoites into the hemolymph. The sporozoites invade the salivary glands and are injected into a new vertebrate host when an infected female takes a second blood meal.

In *Anopheles stephensi*, midgut invasion of *Plasmodium berghei* ookinetes takes place around 24 h after blood feeding and increases the expression of nitric-oxide synthase (NOS) as revealed by immunofluorescence (1) and increased NADPH-dependent nitroblue tetrazolium reduction activity (2). NOS catalyzes the formation of nitric oxide (NO), a highly reactive and toxic molecule (3–5). NO is unstable and reacts readily with other molecules, generating multiple reactive nitrogen intermediates. Peroxynitrite is formed by a rapid reaction between NO and a superoxide anion and readily nitrates proteins in *vivo* (6, 7). Peroxynitrite has also been proposed to be the major mediator of protein nitration in *vivo* (8, 9). NO production plays an important role limiting ookinete infection in the mosquito midgut, as the administration of N-[nitro-arginine methyl ester (L-NAME), a NOS inhibitor, results in a 2-fold increase in the number of developing oocysts (2).

Previous studies indicate that in anophelines *P. berghei* invasion causes irreversible damage leading to cell death (1, 10). Some of the observed changes include loss of microvilli, genome fragmentation, nuclear picnosis (1), and activation of caspases (10). The damage inflicted by parasite invasion is repaired by “budding off” the damaged cells into the midgut lumen through an actin ring-mediated restitution mechanism (1). Invasion of *Plasmodium gallinaceum* ookinetes also damages *Aedes aegypti* midgut cells and results in activation of caspases and cell death (11).

Based on these studies using the *A. stephensi*-*P. berghei* model system, we proposed the “time bomb model” of ookinete midgut invasion, which states that cell invasion triggers a series of toxic reactions (a “bomb”) that leads to cell death and is also potentially toxic to the parasite (1, 12). The model predicts that ookinete survival would depend on the parasite migrating out of the cell before the bomb detonates. In the present study we investigate the biochemistry of the reactions generating the toxic products mediating these defense responses. Our data indicate that in *A. stephensi* and *A. gambiae*, *P. berghei* ookinetes trigger tyrosine nitration as a two-step reaction in which NO generation by NOS is followed by local induction of peroxidase and probably also oxidase enzymes. Peroxidase induction appears to be the rate-limiting step to...
generate highly reactive nitrogen dioxide, which is predicted to mediate tyrosine nitration and to play a critical role in the parasite invasion. To our surprise, only some of the invaded parasites protruded and exhibited high NOS levels (Fig. 1, A. stephensi) as described previously (13). In all the studies, mice having 3–4 exflagellations/field under ×40 objective were used to infect mosquitoes. Blood-fed infected and control mosquitoes were kept at 21 °C in a humidified environment, unless otherwise stated.

**Midgut Immunofluorescence Stainings—**Mosquito midgut immunostainings were performed as described previously (1). Briefly, midguts from freshly fed infected or control mosquitoes were dissected, fixed for 1 min in 4% paraformaldehyde, and opened longitudinally in phosphate-buffered saline (PBS, pH 7.2) to remove the bolus contents. Clean, opened tissues were fixed for 1 h with 4% paraformaldehyde in PBS at room temperature and permeabilized with PBT solution (1% BSA, 0.1% Triton X-100 in PBS) for 2 h at room temperature. Midguts were incubated overnight with the primary antibodies (1:500 dilution in PBT) at 4 °C and at 4 h at room temperature with Cy3- or Cy5- (Amersham Biosciences), or Alexa488-conjugated secondary antibodies (1:500 dilution in PBT). ToPro3 (Molecular Probes) was used to visualize DNA by confocal microscopy. The tissues were washed and mounted in Vectashield™ (Vector Laboratories, Inc.) containing 4',6-diamidino-2-phenylindole to counter stain the nuclei. Immunostainings were analyzed by fluorescence microscopy. The final images were obtained during scanning using confocal microscopy with a Fluoview system and software (Olympus) or regular light and fluorescence microscopy (Olympus) with a color digital camera. The following commercially available antibodies were used: Universal anti-NOS rabbit polyclonal antibody (Affinity Bioreagents, Inc., catalog no. PA1-039) and mouse anti-nitrotyrosine monoclonal antibody (Calbiochem, catalog no. 457823). Anti-Pbs21 monoclonal antibodies were kindly provided by Dr. Roberto Sinden, and anti-AgSRP/N10 rabbit antisera was provided by Dr. Alberto Danielli.

3,3'-Diaminobenzidine (DAB) Activity of the Midgut Tissue—Control or infected blood-fed midguts were dissected 24 h postfeeding, fixed for 1 min in paraformaldehyde, and opened longitudinally to remove the blood meal. For direct DAB stainings, midguts were fixed in 0.5% glutaraldehyde, washed, and fixed at room temperature with Cy5- (Amersham Biosciences), or Alexa488-conjugated secondary antibodies (1:500 dilution in PBT). ToPro3 (Molecular Probes) was used to visualize DNA by confocal microscopy. The tissues were washed and mounted in Vectashield™ (Vector Laboratories, Inc.) containing 4',6-diamidino-2-phenylindole to counter stain the nuclei. Immunostainings were analyzed by fluorescence microscopy. The final images were obtained during scanning using confocal microscopy with a Fluoview system and software (Olympus) or regular light and fluorescence microscopy (Olympus) with a color digital camera. The following commercially available antibodies were used: Universal anti-NOS rabbit polyclonal antibody (Affinity Bioreagents, Inc., catalog no. PA1-039) and mouse anti-nitrotyrosine monoclonal antibody (Calbiochem, catalog no. 457823). Anti-Pbs21 monoclonal antibodies were kindly provided by Dr. Roberto Sinden, and anti-AgSRP/N10 rabbit antisera was provided by Dr. Alberto Danielli.

3,3',5,5'-Tetramethylbenzidine (TMB) Peroxidase Assay—Peroxidase assays using TMB as a substrate were performed following the manufacturer’s instructions (Kirkgegaard & Perry Laboratories, Inc.). Briefly, five uninfected or infected blood-fed midguts were fixed for 1 h at room temperature in PBT containing 4% paraformaldehyde and 1% glutaraldehyde, washed, transferred to 100 μl of a TMB/H2O2 solution, and trituated. After a 10-min incubation at 37 °C in the dark, the midgut tissue was removed by centrifugation and the reaction stopped by adding 1 ml of a stop solution. The amounts (10, 20, and 40 units/ml) of bovine liver catalase (EC 1.11.1.6, Sigma, catalog no. C3155) was determined using the TMB assay as described above, incubating the reactions for 5 min at 37 °C in the dark. The levels of AT activity from midgut homogenates or commercial sources in the presence of 10 μg/ml actinomycin D, using a Hemotek artificial feeder (Discovery Workshop). Midguts were dissected 8 h postfeeding, longitudinally to remove the bolus content, and fixed in 0.5% glutaraldehyde solution at room temperature for 10 min. DAB staining to detect peroxidase activity was carried out in the presence of 10 mg/ml AT (catalase inhibitor) as described above.

Reverse Transcription-PCR Analysis—Poly(A) mRNA was isolated from a group of 20 A. gambiae mosquito midguts 28 h after feeding using Oligotex®-dT beads (QIAGEN), following the manufacturer’s instructions. First strand cDNA was synthesized by using random hexamers and Superscript II (Invitrogen). For the expression studies, PCRs were performed by using 20 pmol of each primer in 50-μl reactions and AmpliTaq (PerkinElmer Life Sciences) with standard buffer conditions (1.5 mM MgCl2). DNA was denatured initially for 3 min at 94 °C followed by 24 cycles of amplification (1 min denaturation at 94 °C, 1 min at the annealing temperature of the specific primer pair, 1 min extension at 72 °C) and a final 10-min extension at 72 °C. For information regarding the protein identification number, primer pair sequence, and reverse transcription-PCR product see the online supplemental material. Amplification of the ribosomal protein gene S7 (14) using primers 5′-GGCGATCATCATCATCAGGC-3′ and 5′-GATGCTGCTGCAACTTCGG-3′ (461 bp) provided the internal control for the amount of cDNA template used in the PCR reactions. The PCR products were analyzed by agarose gel electrophoresis and photographed.
rall difference we took advantage of the fact that single ookinetes often migrate laterally, invading two or more adjacent epithelial cells, as revealed by Pbs21 trails left behind during migration (Pbs21 is a glycosylphosphatidylinositol-anchored surface protein continuously released by migrating *P. berghei* ookinetes), cell protrusion, loss of microvilli, and NOS induction (1). Sequential lateral invasions of *P. gallinaceum* ookinetes in *A. aegypti*, and of *P. berghei* in *A. gambiae* and *A. stephensi* midgut cells, have been observed directly *in vivo* by video microscopy (11) and real-time confocal microscopy (10), respectively. Cells invaded sequentially provide information regarding the relative timing of the cellular responses to the parasite, as more time has elapsed since the invasion of the first than the second, the second than the third cell, and so forth. The location of the ookinite was determined by light microscopy; it is shown in the Fig. 1 as an asterisk and indicates the last (and thus the most recently) invaded cell (Fig. 1, B and C and F and G). We consistently found that, at the time when all cells invaded by the same parasite have already protruded to the lumen and increased NOS expression, tyrosine nitration appeared with a time lag and was often confined to the first cell invaded by the parasite (Fig. 1, B and C). Cells in advanced stages of degeneration, in the process of “budding off” from the midgut epithelium, are heavily nitrated (Fig. 1D). These results indicate that induction of NOS expression is necessary but not sufficient to mediate tyrosine nitration. Occasionally, highly nitrated degenerate cells are negative for NOS staining (Fig. 1C). This could be due to either a decrease in NOS expression or to chemical damage of the epitopes recognized by the anti-NOS antibody.

AgSerpin10 (AgSRPN10), a serine protease inhibitor, is highly induced in *A. gambiae* midguts in response to parasite invasion (15). AgSRPN10 undergoes dramatic changes in subcellular localization and protein expression in a sequential manner (16), and thus, it also provides some information regarding the timing of tyrosine nitration. Healthy uninfected cells express low levels of AgSRPN10 predominantly in the nucleus and are always negative for tyrosine nitration (16) (Fig. 1E). Parasite invasion immediately triggers translocation of the protein from the nucleus to the cytoplasm, which is followed by the induction of high expression levels (16). Tyrosine nitration is first detected when the cytoplasmic levels of AgSRPN10 begin to increase (Fig. 1E) and becomes stronger as AgSRPN10 expression further increases (Fig. 1, F and G). All cells positive for nitrotyrosine staining express high AgSRPN10 levels; however, cells with high AgSRPN10 expression are not always nitrated (Fig. 1, F and G). These data indicate that protein nitration takes place during the later stages of the cell death process triggered by parasite invasion.

**Ookinete Invasion Induces a Glutaraldehyde-resistant Peroxidase Activity**—The classic model of peroxynitrite-mediated tyrosine nitration in vertebrates has been challenged based on kinetic studies in murine RAW 264.7 macrophage cells (17). Both NO and superoxide anion levels were found to increase following immune stimulation with interferon-γ/yilipopolysaccharide or interferon-γ/zymosan A, but they rapidly decreased.
to base-line levels several hours before tyrosine nitration could be detected. NO formation resulted in nitrite accumulation, which was proposed to serve as a substrate for a myeloperoxidase (MPO)-mediated tyrosine nitration reaction (17). Experiments using multiple distinct models of acute inflammation with eosinophil peroxidase (EPO) and MPO knock-out mice indicate that leukocyte peroxidases participate in nitrotyrosine formation in vivo (18). In some models, MPO and EPO played a dominant role, accounting for the majority of nitrotyrosine formed. However, in other leukocyte-rich acute inflammatory models, neither MPO nor EPO contributed to nitrotyrosine formation, implying the existence of alternative nitration pathways (18).

Based on the vertebrate data we decided to test the hypothesis that protein nitration of invaded midgut cells was mediated by ookinete-induced peroxidase(s). Midguts of females fed on healthy (Ctl) or malaria-infected (Inf) mice were fixed briefly with glutaraldehyde and assayed for peroxidase activity using DAB and hydrogen peroxide as substrates (Fig. 2A). Within a few minutes of incubation some of the malaria-infected cells protruding into the lumen stained very strongly with DAB (Fig. 2A, right panel), whereas no staining was detected in control samples incubated for the same amount of time (Fig. 2A, left panel). The cells positive for DAB staining are in close association with invading ookinetes. Furthermore, when two adjacent cells are invaded by the same parasite, the peroxidase activity is usually much higher in the cell that was invaded first (Fig. 2, B and C), in a pattern very similar to that described above for tyrosine nitration (Fig. 1, B and C). When P. berghei-infected females were kept at 28 °C, a non-permissive temperature for ookinete development, neither cells protruding into the midgut lumen nor DAB staining was observed (data not shown), implying that these two events are triggered by ookinete invasion.

As both catalase and peroxidase activities can give rise to a positive DAB reaction (19), the relative contribution of these two enzymes was evaluated by performing stainings in the presence of specific inhibitors. The addition of AT, a catalase inhibitor, had no effect, whereas sodium azide, a peroxidase inhibitor, completely abolished the reaction. To confirm the induction of peroxidase activity in response to malaria infection, the activity was also determined in midgut homogenates by performing a colorimetric assay using TMB, a peroxidase-specific chromophore, and H$_2$O$_2$ as substrates. parasite infection resulted in a marked increase in peroxidase activity when homogenates from midguts that had been fixed with a mixture of glutaraldehyde and paraformaldehyde were used in the assay (Fig. 2D). However, no difference in total peroxidase activity could be observed between control and infected samples when unfixed tissues or tissues fixed only with paraformaldehyde were used (data not shown). This observation suggests that parasite-induced peroxidase(s) is more resistant to fixation than other peroxidases constitutively ex-

![Fig. 2. DAB staining of P. berghei-infected A. stephensi midguts 24 h after feeding.](image)

A, non-infected (Ctl) and infected (Inf) midguts were stained using DAB as a substrate (brown). Some invaded cells protruding into the midgut lumen stained very strongly, whereas healthy cells and uninfected midguts were negative for DAB staining. The bar represents 20 μm. B and C, midguts were double stained for the ookinete surface protein Pbs21 (red immunofluorescence) and DAB. The bar represents 10 μm. B, invading ookinetes colocalize with DAB-positive (brown) midgut cells (left panel). When two adjacent cells are sequentially invaded (right panel) only the first invaded cell exhibits strong DAB staining in a pattern similar to that observed for nitrotyrosine staining in Fig. 1. C, the catalase inhibitor 3-aminotriazole (AT) did not affect DAB staining, whereas sodium azide (AZ), a peroxidase inhibitor, completely abolished the reaction. D and E, peroxidase activity in homogenates from midguts collected 24 h postfeeding. The bars represent the mean value of the absorbance at 450 nm ± S.E., n = 3. D, peroxidase activity using the TMB substrate (specific for peroxidase) in control (C) and malaria-infected (I) midgut homogenates. E, effect of sodium azide (closed circles) and 3-aminotriazole (open circles) on the peroxidase activity of homogenates from malaria-infected midguts. F, effect of increasing concentrations of commercial catalase on the catalytic activity of a commercial peroxidase. G, effect of increasing concentrations of 3-aminotriazole on the inhibitory effect of commercial catalase (Cat) on peroxidase activity.
Inhibitor, was added to negative control samples. After the reaction, BSA samples were subjected to denaturing SDS-PAGE and Western blot analysis (WB) using the same anti-nitrotyrosine monoclonal antibody as in the immunofluorescence experiments. Duplicate samples were stained with Coomassie blue (CB) following SDS-PAGE. A commercial peroxidase (Per.) was used as positive control, and NaN₃, a peroxidase inhibitor, was added to negative control samples.

Presented here were performed in midgut tissues fixed previously with a mixture of glutaraldehyde and paraformaldehyde (see “Experimental Procedures” for details). As expected, sodium azide had a strong inhibitory effect on this inducible peroxidase activity in contrast to the catalase inhibitor AT, which had the opposite effect, slightly increasing peroxidase activity (Fig. 2E). This activity enhancement is probably because of a competition between catalase and peroxidases present in the midgut homogenate for hydrogen peroxide, a common substrate. We confirmed that the addition of commercial catalase to a commercial peroxidase did have an inhibitory effect (Fig. 2F) that could be alleviated by inhibiting catalase activity with AT (Fig. 2G).

The Ookinete-induced Peroxidase Activity Can Mediate Protein Nitration—An in vitro nitration assay was performed to determine whether the ookinete-induced peroxidase activity could mediate protein nitration. Midgut homogenates from infected or non-infected mosquitoes were incubated with BSA in the presence of NaN₃ and H₂O₂. Following incubation, BSA was subjected to SDS-PAGE and electroblotted, and nitrotyrosine was detected by Western blot analysis using the same anti-nitrotyrosine antibody used for immunofluorescence staining. The BSA sample incubated with infected midgut homogenate had a higher level of nitrotyrosine staining relative to the control uninfected samples (Fig. 3). Tyrosine nitration required all components to be present, a source of peroxidase activity, sodium nitrite, and hydrogen peroxide; the removal of any of them completely inhibited the reaction. As expected, the addition of sodium azide inhibited nitration mediated either by midgut homogenates or by a commercial peroxidase used as a positive control for the reaction (Fig. 3).

Ookinete-induced Peroxidase Activity in A. gambiae Midguts Correlates with Transcriptional Activation of Several Peroxidase Genes—The invasion of A. gambiae midguts by P. berghei also induced cell protrusion, localized peroxidase activity, and apoptosis of midgut epithelial cells. Four of the five peroxidase genes predicted from the Ensembl A. gambiae genome browser sequence were evaluated by reverse transcription-PCR analysis (data not shown). Six peroxidases (Fig. 4B, left panel) were found to be differentially expressed in response to ookinete invasion; five of them (ENSANGP00000006017, ENSANGP00000019589, ENSANGP00000011001, ENSANGP0000010957, and ENSANGP00000019620) were induced, whereas one (ENSANGP0000001781) exhibited a dramatic reduction in mRNA levels. The ENSANGP00000006017 protein is a dual oxidase (Ag-Duox), predicted to have two functional domains, an N-terminal peroxidase homology domain and a C-terminal domain with homology to the gp91 subunit (the catalytic moiety) of phagocyte NADPH oxidase (Fig. 4B, bottom) (20). Between these two domains, two regions with homology to EF-hand calcium-binding sites can be found (Fig. 4B, yellow ovals). EF-hands are helix-loop-helix motifs that potentially bind calcium (21). Ag-Doux mRNA could not be detected in control samples but was found to be highly abundant in conditions permissive for ookinete development and invasion (Fig. 4B, left panel). Other peroxidases either were not expressed in the midgut (ENSANGP00000012402, ENSANGP00000012538, ENSANGP00000012513, ENSANGP000000593, ENSANGP00000010602, ENSANGP000000185-05, ENSANGP00000019765, and ENSANGP00000017343) or their expression did not change in response to infection with P. berghei (ENSANGP0000000569 and ENSANGP00000021320).

Actinomycin D-induced Apoptosis of Midgut Cells Also Induces Peroxidase Activity—To investigate whether peroxidase induction was a specific response to parasite invasion or part of a more general mechanism of apoptosis, A. stephensi (Fig. 4C) and A. gambiae (data not shown) females were fed a BSA solution containing actinomycin D (Act) and analyzed 8 h post-feeding. Actinomycin D induced apoptosis in both species, as evidenced by nuclear fragmentation into nuclear bodies (Fig. 4C, lower panel) and the presence of cells budding off into the midgut lumen. This chemically induced apoptosis also resulted in strong peroxidase-mediated DAB staining (Fig. 4C, upper panel), indicative of glutaraldehyde-resistant peroxidase activity and suggests that this response is part of a more general apoptotic mechanism of midgut epithelial cells. Four of the five ookinete-induced A. gambiae peroxidases (including Ag-Duox) also showed increased mRNA expression levels following actinomycin D treatment in A. gambiae (Fig. 4B, right panel). Peroxidase ENSANGP00000001781 mRNA levels also decreased markedly in response to both parasite invasion and actinomycin D. These results demonstrate that at least five peroxidases are differentially expressed during apoptotic responses of midgut epithelial cells.

**DISCUSSION**

Factors Determining the Rate of Tyrosine Nitration—Nitrite, the primary metabolic end product of nitric oxide, can be oxidized by the heme peroxidases in the presence of hydrogen peroxide (22). Based on the chemical analyses of the intermediate products of MPO activity on nitrite and hydrogen peroxide as substrates, nitrite, a powerful oxidizing species, has been proposed to be the most likely reaction product, and a second pathway that would generate peroxynitrite, although possible, was thought to play a minor role (23). Recent experiments argue that both pathways (generating nitrite or peroxynitrite) operate simultaneously and can both play significant roles in vivo (24).

The reactions proposed to mediate and regulate in vivo nitration of midgut epithelial cells are shown schematically in Fig. 5. NOS generates NO, an unstable product that is readily converted to nitrite, and previous work has demonstrated that nitrite accumulates in malaria-infected mosquitoes (2). The proposed reactions (Fig. 5) predict that a localized increase in peroxidase activity is required for nitration to proceed. Our results indicate that there is a time lag between NOS expres-
sion and protein nitration in ookinete-invaded cells (Fig. 1, A–C), suggesting that peroxidase induction could be the rate-limiting step. In addition to peroxidase, the nitration reaction also requires the local accumulation of high levels of hydrogen peroxide. In human macrophages this is achieved by the induction of an NADPH-dependent oxidase that uses an electron from cytosolic NADPH to reduce extracellular oxygen to a superoxide anion (25). The local induction of Ag-Duox (EN-SANGP00000006017) could play a critical role as the source of high local levels of superoxide anion. CuZn superoxide dismutase expression is highly induced in the midgut 24 h after blood feeding independent of malaria infection (26) and could catalyze the dismutation of superoxide anion to hydrogen peroxide. Ag-Duox has homologues in Drosophila spp., humans, and Caenorhabditis elegans. In C. elegans Ce-Duox catalyzes the cross-linking of tyrosine residues, which stabilizes the cuticular extracellular matrix (20). A dual enzyme with both NADPH oxidase and peroxidase activities has also been described in salivary gland homogenates of Anopheles albimanus (27, 28).

Our data indicate that the midgut epithelium has a remarkable ability to localize tyrosine nitration to individual cells, a process that is essential to prevent the spread of toxic products and apoptosis to adjacent healthy cells. This is in part accomplished through the localized expression of peroxidases and presumably oxidase(s) in the invaded cells. However, a peroxidase that is very abundant in healthy control midguts (EN-SANGP00000001781) responds very differently, dramatically down-regulating mRNA expression following ookinete invasion. This response suggests that cell-to-cell signaling could be taking place. The transient removal of peroxidase activity from healthy cells could prevent the formation of nitrogen dioxide, thus preventing tyrosine nitration. This enzyme could also

**Fig. 4.** A, DAB staining (brown) of non-infected (Ctl) and infected (Inf) A. gambiae midguts 24 h after feeding. Parasites are stained with anti-Pbs21 (red) antibody. B, induction of peroxidase genes in A. gambiae midguts by P. berghei infection or actinomycin D feeding. Females fed on normal (C) or infected (I) mice were maintained at either a permissive (21 °C) or non-permissive (28 °C) temperature for 28 h. The females fed on BSA (−) or 10 μg/ml actinomycin D (+) were kept at 28 °C for 8 h. Midguts were dissected, and a semi-quantitative reverse transcription-PCR was carried out by using specific primers for each gene as described under “Experimental Procedures.” The ribosomal protein S7 gene was used as internal control for the amount of cDNA template. The bottom diagram illustrates the organization of the predicted Ag-Duox protein. The N terminus (orange) has homology to peroxidases, whereas the C terminus has homology to the catalytic domain (gp91 domain) of NADPH oxidase. The blue bars represent the putative transmembrane hydrophobic regions and the black bar the conserved NADPH-binding site, which contains the canonical nucleotide-binding motif (GXXGXXP). The regions showing homology to EF-hand calcium-binding sites are indicated by the yellow ovals. The same organization and conserved motifs have been described previously for other Duox homologues (20). C, DAB staining of the BSA-fed midguts (Ctl) or 10 μg/ml actinomycin D-fed midguts (Act. D). The 8-h fed glutaraldehyde-fixed midguts were incubated with DAB (brown, upper panel), and their nuclei were counterstained for 4′,6-diamidino-2-phenylindole (blue, lower panel). The bar represents 5 μm.
explain the constitutive peroxidase activity observed in unfixed control midguts. Temporal-spatial expression analysis of the protein product of this gene would be necessary to confirm this hypothesis.

The fact that we frequently observed strong nitration only in the first cell when a single parasite has invaded several adjacent cells could be interpreted in two different ways: there could be something different about the first invaded cell itself or in the injury response when the apical surface of the cell is damaged or, alternatively, all invad cells eventually undergo nitration and apoptosis, but the process takes place sequentially, one cell at a time. We favor the second interpretation, as we have found previously that although many healthy oocysts are observed 48 h postinfection, the cells overexpressing NOS and protruding into the lumen can no longer be found at this time, suggesting that they have all been shed into the midgut lumen as part of the epithelial repair mechanism (1). Detailed analysis of AgSRPN10 translocation and expression also indicates that cell degeneration is a gradual process that takes place sequentially as the parasite traverses adjacent cells (16).

Our observation that several peroxidases from A. gambiae are transcriptionally activated when apoptosis is induced in midgut epithelial cells suggests that besides their role in immunity these enzymes also participate in general apoptotic responses. MPO has been implicated in hydrogen peroxide-induced apoptosis of HL-60 human leukemia cells (29). Incubation of HL-60 cells with hydrogen peroxide resulted in dose-dependent stimulation of caspase-3 activity, DNA fragmentation, and morphological changes associated with apoptosis that were inhibited by pre-incubation of the cells with a MPO-specific inhibitor (29). Moreover, agonist-induced apoptosis of neutrophils from MPO-deficient mice were found to be significantly slower than in wild type cells (30).

We observed strong tyrosine nitration in those parasite-invaded cells in advance stages of apoptosis (Fig. 1). Eosinophil peroxidase has been found to generate nitrogen dioxide in the presence of nitrite and hydrogen peroxide and to induce cell death of lung epithelial cells through activation of c-Jun-N-terminal kinase (JNK) (31). Furthermore, exposure to lipopolysaccharide leads to activation of JNK in Drosophila spp (32), and a putative JNK orthologue is present in the A. gambiae genome (ENSANGP00000025193). Thus the potential role of this signal transduction pathway in tyrosine nitration warrants further investigation.

Immunity and apoptosis are two tightly linked processes as the highly reactive chemicals used to attack pathogens are also toxic to the cells mounting the defense response. Precise compartmentalization of the catalytic activities generating reactive oxygen species and reactive oxygen intermediates and the activity of detoxification enzymes such as catalase and superoxide dismutases are required to prevent cell damage that could lead to wide-spread apoptosis. In vitro stimulation of the murine macrophage cell line (RAW 264.7) with lipopolysaccharide and/or interferon-γ induced strong endogenous NO production and apoptosis. The surviving cells (10–50% depending on the experiment) were selected and found to be capable of surviving when further exposed in vitro to an apoptosis-inducing dose of the NO donor compound diethylenetriamine nitric oxide. These resistant cells expressed increased steady-state levels of man...
Eukaryotic protein nitration, Cu/Zn superoxide dismutase, and catalase mRNAs (130–200%) and enzymatic activities, as well as increased intracellular glutathione levels (33).

Revised Model of Ookinete Invasion of Midgut Cells—Fig. 6 shows an updated version of the previously proposed time bomb model of oocYTE invasion (1). Parasite invasion results in the induction of NOS and the production of NO, which is thought to convert to nitrite and accumulate in the infected female. In a second step, the induction of high levels of peroxidase activity (and presumably oxidase activity, as well) takes place in the invaded cells and mediates tyrosine nitration. After this second step, extensive cell degeneration is observed. There is a time delay between NOS and peroxidase induction so that, in susceptible mosquito strains, ookinetes have already exited the invaded cell when tyrosine nitration occurs. The relative timing of parasite migration and the activation of nitration are predicted to play a critical role in parasite survival. As we have proposed previously, parasite invasion triggers a time bomb-like response so that the oocYTE has a limited time window (before nitrogen dioxide is produced) to escape unharmed. Based on this model, high levels of hydrogen peroxide are expected to accelerate nitrogen dioxide formation and tyrosine nitration. This is particularly provocative, as we have recently reported that an A. gambiae strain (L35) that melanizes the parasites, and is thus refractory to malaria infection, has constitutively high hemolymph levels of hydrogen peroxide, which further increase 24 h postfeeding (26). This refractoriness could be explained by an accelerated rate of nitration or by some other oxidation reaction that damages the parasite surface, making it “visible” to the mosquito immune system and triggering activation of the melanization cascade. The cell biology of oocYTE invasion in this refractory mosquito strain is currently under investigation.

The fact that this two-step tyrosine nitration mechanism is also observed in mosquitoes suggests that this is an ancient mechanism that existed before insects and humans diverged. It probably evolved to precisely localize and circumscribe the mechanism that existed before insects and humans diverged. It also observed in mosquitoes suggests that this is an ancient mechanism that existed before insects and humans diverged. It also observed in mosquitoes suggests that this is an ancient mechanism that existed before insects and humans diverged.

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