

Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*

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Studies in mammals uncovered important signaling roles of nitric oxide (NO), and contributions to innate immunity. Suggestions of conservation led us to explore the involvement of NO in *Drosophila* innate immunity. Inhibition of nitric oxide synthase (NOS) increased larval sensitivity to gram-negative bacterial infection, and abrogated induction of the antimicrobial peptide Diptericin. NOS was up-regulated after infection. Antimicrobial peptide reporters revealed that NO triggered an immune response in uninfected larvae. NO induction of Diptericin reporters in the fat body required *immune deficiency (imd)* and *domino*. These findings show that NOS activity is required for a robust innate immune response to gram-negative bacteria, NOS is induced by infection, and NO is sufficient to trigger response in the absence of infection. We propose that NO mediates an early step of the signal transduction pathway, inducing the innate immune response upon natural infection with gram-negative bacteria.

[*Keywords:* *Drosophila*; nitric oxide; signaling; innate immunity; hemocyte; Relish]

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The innate immune system is an ancient first line of defense against foreign organisms (Hoffmann et al. 1999). In contrast to the genetic rearrangements and clonal selection processes that underlie adaptive immunity, innate immunity relies on the functions of germ-line encoded gene products. Nitric oxide (NO) is a highly reactive molecule with innate immune functions as well as roles in responses to hypoxia and CNS development (Dawson et al. 1991; Bredt and Snyder 1994; Gibbs and Truman 1998; Wingrove and O'Farrell 1999; Bogdan 2001). NO is produced in mammalian macrophages by a nitric oxide synthase isoform (iNOS/NOS2), which is strongly up-regulated following infection (MacMicking et al. 1997). Macrophages also produce high quantities of superoxide by NADPH oxidase following pathogen detection (Ding et al. 1988). The highly toxic molecules NO, superoxide, and their derivatives are believed to play an important role in destroying invading microorganisms (Nathan and Shiloh 2000). In seeking to develop *Drosophila* as a model for studying mechanisms modulating and directing this toxicity, we found that NO plays a signaling role in the induction of immune responses in *Drosophila* larvae to gram-negative bacteria.

Metazoan defenses against foreign organisms are commonly induced by pathogen detection. Components of

the innate immune system recognize chemical structures that are hallmarks of microorganisms and are not found on host cells (Akira et al. 2001). These pathogen-associated molecular patterns (PAMPs) include β -1,3-glucan of fungi, peptidoglycan and lipopolysaccharides of bacteria, and phosphoglycan of parasites. Widespread PAMPs are recognized by pattern recognition receptors (PRRs). PRR activation by binding of cognate PAMPs initiates host-signaling cascades that start defense responses.

Toll (Tl) signal transduction, which was originally identified on the basis of its role in dorsal/ventral patterning of the *Drosophila* embryo, is now recognized as having an evolutionarily conserved role in pathogen detection and induction of immune responses (Rosetto et al. 1995; Lemaitre et al. 1996; Nicolas et al. 1998; Qiu et al. 1998; Kopp and Medzhitov 1999; Aderem and Ulevitch 2000; Tauszig et al. 2000; Michel et al. 2001; Ooi et al. 2002; Tauszig-Delamasure et al. 2002; Underhill and Ozinsky 2002). The *Drosophila* Tl pathway is particularly responsive to fungi and gram-positive bacteria, which trigger processing and activation of the Tl ligand, Spaetzle (Spz; Levashina et al. 1999). Tl receptor-dependent signaling activates two NF- κ B-related transcription factors, Dorsal (Dl) and Dorsal-related immunity factor (Dif), and expression of defense genes such as those encoding antimicrobial peptides (Lemaitre et al. 1995b; Meng et al. 1999).

In *Drosophila*, a pathway that is independent of Tl, the Immune Deficiency (Imd) pathway, is activated in re-

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sponse to gram-negative bacteria. A putative peptidoglycan recognition protein (PGRP-LC) contributes to this pathway (Choe et al. 2002; Gottar et al. 2002; Ramet et al. 2002). Although there are uncertainties regarding its specificity, PGRP-LC has attributes of a PAMP and it acts upstream of the death-domain-containing protein Immune deficiency (Imd). Imd signaling proceeds through the MAP kinase kinase kinase homolog, dTAK1, the caspase, dredd, and an I- κ -kinase to culminate in the proteolytic activation of the NF- κ B family member Relish (Rel; Lemaitre et al. 1995a; Dushay et al. 1996; Wu and Anderson 1998; Hedengren et al. 1999; Kim et al. 2000; Silverman et al. 2000; Stoven et al. 2000; Georgel et al. 2001; Lu et al. 2001; Vidal et al. 2001). Mutants lacking Rel are highly sensitive to infection with gram-negative bacteria (Hedengren et al. 1999).

Two protocols have been established for investigating responses of *Drosophila* larvae to infection. Piercing the larval cuticle with a needle soiled with pathogen triggers a robust induction of the innate immune response. Whereas this septic injury protocol has been used successfully to define the outlines of the signaling cascades that induce host responses to infection, it has a disadvantage. The trauma of injury weakly activates immune responses even without bacteria, thus eroding the specificity of the response. A recently devised natural infection protocol activated the Imd pathway by briefly feeding larvae on concentrated slurries of a gram-negative bacteria such as *Erwinia carotovora carotovora* (Ecc; Basset et al. 2000).

We present evidence that NO makes crucial contributions to the Imd pathway that activates Rel. Pharmacological inhibition of NOS increases larval and adult susceptibility to septic and natural infection with gram-

negative bacteria and compromises production of antimicrobial peptides. Treatment of uninfected larvae with NO donors is sufficient to activate antimicrobial peptide production. Genetic experiments demonstrate that one action of NO is either upstream of Imd or in a parallel pathway required for Rel activation of innate immune responses in the fat body. Ingestion of bacteria is followed by induction of NOS in the gut and in hemocytes. NO-mediated induction of the antimicrobial peptide Dipterin in the fat body was blocked in *domino* mutants. We propose that NO is an innate immune signal that acts early in a multi-tiered cascade in which sentinel tissues detect gram-negative bacteria and recruit other tissues to host defense.

Results

NOS contributes to pathogen destruction

We tested whether inhibition of NOS influences the ability of larvae to survive a bacterial infection. *Drosophila* has a single NOS homolog for which there are no available mutant alleles (Regulski and Tully 1995; Stasiv et al. 2001). To pharmacologically inactivate NOS, we treated larvae with the NOS-inhibitory arginine analog N ω -Nitro-L-Arginine-Methyl-Ester (L-NAME) and used the septic injury and natural infection models to infect third instar larvae with the *Drosophila* pathogen *Ecc* (Fig. 1A,B, respectively). In the absence of infection, treatment with L-NAME or the inactive D-enantiomer had little impact on eclosion/survival. Furthermore, injury alone did not significantly compromise eclosion/survival. In contrast, L-NAME, but not D-NAME treatment dramatically reduced the ability of larvae to sur-

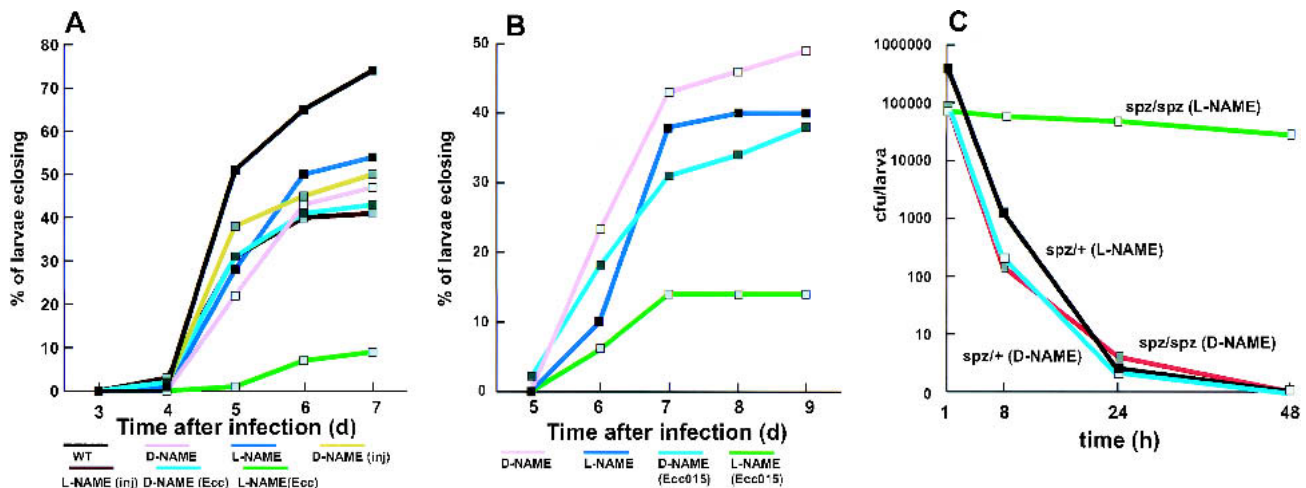


Figure 1. NOS activity contributes to pathogen destruction. (A,B) The ability of third instar wild-type larvae to survive septic (A) and natural (B) infections and eclose. In both cases, inhibiting NOS by feeding L-NAME compromised the ability of the larvae to survive infection with *Erwinia carotovora* (L-NAME Ecc). In contrast, larvae survived infection when treated with a control isomer of the inhibitor (D-NAME Ecc) as well as enduring control treatments such as treatment with the drugs with or without injury (inj). (C) The bacterial titer at different times after Ecc015 natural infection of third instar larvae shows that host killing of bacteria is promoted by Spz-dependent and NOS-dependent processes. Whereas *spz* heterozygous larvae treated with L-NAME or D-NAME and *spz* mutants treated with D-NAME successfully destroyed invading bacteria, inactivation of Spz by mutation and NOS by L-NAME prevented host elimination of bacteria.

vive a subsequent *Ecc* septic infection (Fig. 1A,B). Similar effects were observed with adult flies subjected to a septic infection (data not shown). We conclude that inactivation of NOS impairs the ability of *Drosophila* to combat subsequent infections by two different routes.

To distinguish contributions of NOS to the Imd-dependent and Spz-dependent defense pathways, we tested the consequence of NOS inhibition in mutant strains incapable of activating one or the other pathway. D-NAME had no effect and L-NAME only a modest effect on the ability of *spz* heterozygous larvae to eliminate bacteria (Fig. 1C; data not shown). In contrast, homozygous *spz* mutant larvae exhibited a remarkably different response to feeding D-NAME and L-NAME; at 24 h after infection, bacterial titer was 10,000 times higher in *spz* mutants fed L-NAME (Fig. 1C). This result indicates that L-NAME severely compromises Spz-independent host defenses. The major defense against gram-negative bacteria such as *Ecc* is thought to be mediated via the Spz-independent Imd pathway, which is thus a candidate target for the action of L-NAME.

Bacterial titer remained high in *imd* mutants whether or not they were fed L-NAME (data not shown). This is in accord with the possibility that L-NAME acts on the Imd pathway, but we cannot assess from this result whether NOS regulates any Imd-independent contributions to host defense, because such pathways do not ap-

pear to confer any significant resistance to our infection protocol.

NO is a signaling molecule in the Imd/Rel pathway

The emphasized role of NO in the mammalian host defense is its involvement in pathogen killing. Studies in insect systems have also indicated a role for NOS in eliminating pathogens (Dimopoulos et al. 1998; Luckhart et al. 1998; Han et al. 2000), but there are suggestions that its action might include induction of immune responses (Nappi et al. 2000; Imamura et al. 2002). For this reason, we tested whether NO might have a role as an upstream regulator of the immune response by testing the influence of L-NAME treatment on induction of the antimicrobial peptide Diptericin (Dipt). Induction of Dipt in the fat body after a natural or septic infection is strictly dependent on Imd and Relish (Lemaitre et al. 1995a; Hedengren et al. 1999). We followed Dipt induction using a reporter construct in which GFP expression is controlled by the *Dipt* promoter (*Dipt-GFP*). This reporter mimics the infection-induced expression of the endogenous *Dipt* gene (Tzou et al. 2000).

No GFP was observed in *Dipt-GFP* larvae in the absence of infection. A large fraction (79%) of larvae naturally infected with *Ecc* produced GFP in the fat body (Fig. 2B,F) within 16 h. Whereas D-NAME did not compromise this induction (Fig. 2C,F), the fraction of GFP-expressing larvae was dramatically reduced by L-NAME

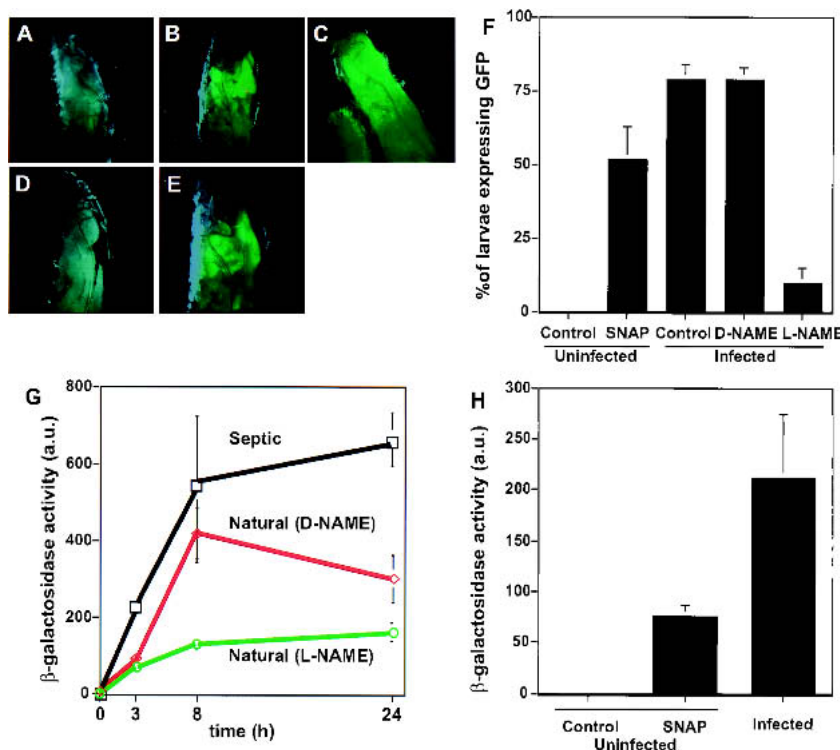


Figure 2. NO signaling activates expression of a *Dipt* reporter. (A–E) GFP fluorescence in the anterior half of larvae that express GFP under the control of the *Dipt* promoter (*Dipt-GFP*). Uninfected larvae do not express the reporter (A, the weak green signal is background autofluorescence), whereas infection with *Ecc* induces reporter activity (B). GFP expression persists in D-NAME-treated naturally infected larvae (C), whereas it is absent in L-NAME-treated naturally infected larvae 16 h postinfection (D). (E) The NO donor SNAP induces GFP in uninfected larvae. (F) Bar graph showing the percentage of larvae expressing GFP 16 h following the different treatments. (G) Graph showing the β -galactosidase activity measured in larval extracts prepared from third instar larvae that express β -galactosidase under the control of the *Dipt* promoter (*Dipt-lacZ*) as a function of time after infection. Whereas D-NAME-fed larvae display a robust β -galactosidase activity 8 h after natural infection, L-NAME-fed larvae exhibit a lower β -galactosidase. The value for each time point is the average of three independent measurements. The high levels of β -galactosidase activity in extracts from *dipt-lacZ* larvae exposed to a septic injury with *Ecc*015 persist somewhat longer than the levels in naturally infected larvae, but

the initial responses differ only slightly. (H) Graph showing β -galactosidase activity in *Dipt-lacZ* larvae prior to treatment with SNAP and 24 h after treatment. Provision of SNAP to uninfected larvae significantly increases β -galactosidase activity. Data are the average of three independent measurements.

treatment (Fig. 2D,F, 10%). Similar effects were seen after natural infection with *Escherichia coli*, although *E. coli* was not as potent as *Ecc* in inducing the reporter (data not shown). Thus, inhibition of NOS greatly reduced infection-dependent expression of the *Dipt* reporter.

To determine whether NO is sufficient to activate *Dipt* production on its own, uninfected *Dipt-GFP* larvae were fed the NO-donor S-Nitroso-N-acetylpenicillamine (SNAP) and examined 16 h later. Exposure to exogenous NO induced GFP in ~50% of the larvae despite the lack of infection (Fig. 2E,F). Control *Dipt-GFP* larvae treated with prereacted SNAP that no longer released NO did not express the reporter (data not shown). Therefore, we conclude that NOS activity is required for *Dipt* expression after natural infection with gram-negative bacteria, and that NO is sufficient to activate *Dipt* expression in the absence of infection. Both infection and SNAP treatment resulted in an initial mosaic pattern of GFP in the fat body (data not shown; Reichhart et al. 1992). Thus, exogenously provided NO not only activates the Imd pathway in the absence of infection, it also recapitulates this spatial feature of *Dipt* expression.

To compare the efficacy of natural and septic infections, and to assess the degree of inhibition of the response by L-NAME, we measured β -galactosidase activity in extracts of treated larvae that express β -galactosidase under the control of the Dipterin promoter (*Dipt-lacZ*). Septic infection produced a slightly faster and more persistent rise in β -galactosidase than natural infection, but this difference was small compared with the suppression of the response upon incubation with L-NAME (Fig. 2G). Note that there is a slow rise in β -galactosidase even in the presence of L-NAME, and that control larvae appear to be down-regulating the response within 24 h (Fig. 2G). We also quantified the β -galactosidase activity of uninfected, SNAP-treated *Dipt-lacZ* larvae 24 h after feeding SNAP. Exposure to NO led to a clear elevation of β -galactosidase activity in uninfected larvae (Fig. 2H), supporting the results with *Dipt-GFP* (Fig. 2E,F), indicating that NO is sufficient to induce *Dipt* expression in uninfected larvae.

In summary, pharmacological inhibition of NOS inhibits, or greatly retards induction of *Dipt* reporter constructs in response to infection, whereas dietary provision of NO to larvae stimulates *Dipt* production in the absence of infection. We propose that NO contributes to the activation of the Imd/Rel pathway. As L-NAME-treated wild-type larvae eliminate pathogens more effectively than *imd* mutants, we believe that pharmacological inhibition of NOS does not completely inactivate Imd-dependent signaling. In agreement with this, we noticed that a low percentage of L-NAME treated infected *Dipt-GFP* larvae still express GFP upon infection and that, although greatly reduced and delayed, low-level reporter induction occurred in the presence of L-NAME. Nonetheless, the contribution of NOS to gram-negative bacterial destruction is substantial, as L-NAME treated *spz* mutants are greatly impaired in their ability to clear *Ecc* upon natural infection when compared with untreated control *spz* mutant larvae.

NO effects on Drosomycin expression

Whereas *Dipt* is induced strictly via the Imd pathway, Drosomycin (*Drs*) is also induced by the Tl pathway (Lemaitre et al. 1996). To test whether NOS is required for this response, we examined induction of a reporter expressing GFP under the control of the *Drs* promoter (*Drs-GFP*). A few uninfected larvae (7%) exhibited foci of *Drs-GFP* expression, but natural infection with *Ecc* dramatically increased the incidence and extent of GFP expression 16 h post infection (Fig. 3B). The number of larvae exhibiting this infection-induced *Drs* expression was not appreciably affected by D-NAME or L-NAME treatment (88% and 80%, respectively; Fig. 3F). Thus, it appears that inhibition of NOS does not prevent infection-dependent *Drs* induction. Nonetheless, the NO donor SNAP induced GFP in ~50% of uninfected *Drs-GFP* larvae (Fig. 3E,F).

To quantify contributions of NO to *Drs* expression, we prepared extracts from naturally infected larvae that express β -galactosidase under control of the *Drs* promoter (*Drs-lacZ*). Following infection, L-NAME fed larvae had significantly less (by nearly half) β -galactosidase activity than D-NAME fed larvae (Fig. 3G). Thus, it appears that although NOS is not required for all *Drs* reporter activity, NOS activity makes a quantitative contribution. Consistent with a quantitative contribution of NO to the induction, we observed that SNAP mediated a substantial induction of *Drs-lacZ* in uninfected larvae (Fig. 3H). Whereas the most straightforward interpretation of these results is that infection-mediated induction of *Drs* occurs via two pathways (see Discussion), a NOS dependent and independent one, we cannot exclude the possibility that L-NAME only partially blocks a single NO-dependent pathway.

Epistatic relations of NOS and Imd in the Imd/Rel pathway

To test whether NO induction of a *Dipt* reporter involves the Imd pathway, we tested the response in the fat bodies of *imd* mutant larvae. As shown by activity stain for β -galactosidase in dissected fat bodies from *Dipt-lacZ* larvae (Fig. 4), NO induction of the reporter requires Imd function. Reciprocally, overexpression of *Imd*, which activates immune responses (Georgel et al. 2001) and reporter expression (Fig. 4H) in the absence of infection, retained its ability to activate *Dipt-lacZ* expression in the presence of the NOS inhibitor, L-NAME (Fig. 4I). These findings are most simply consistent with action of NO upstream of *Imd* in the signal transduction pathway inducing *Dipt* in the fat body, although it remains possible that NO acts in a parallel pathway.

NOS involvement in hemocyte responses to infection

In addition to the fat body, other cells such as hemocytes produce antimicrobial peptides after infection. To test whether NO impinges upon hemocyte expression of antimicrobial peptides, we naturally infected or SNAP

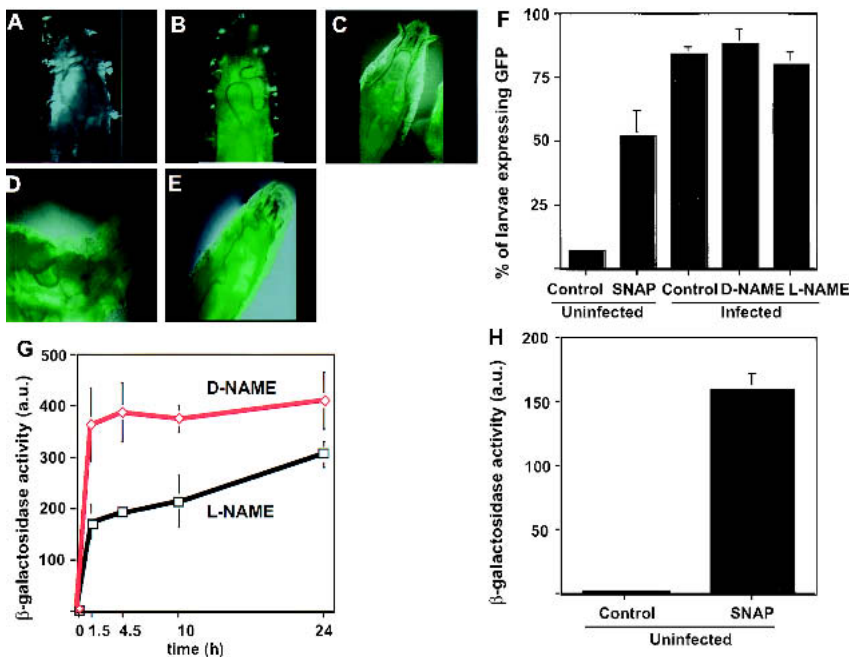


Figure 3. NO activates expression of a *Drs* reporter, but NOS inhibition only partially reduces *Drs* induction after infection. (A–E) GFP fluorescence in the anterior half of larvae that express GFP under the control of the *Drs* promoter (*Drs*–GFP). Most uninfected larvae do not express the reporter (A), whereas larvae naturally infected with *Ecc* do (B). Naturally infected larvae treated with either D-NAME (C) or L-NAME (D) produce GFP, and provision of SNAP to uninfected larvae induces GFP (E). (F) Bar graph showing the percentage of larvae expressing GFP following the different treatments. (G) Graph showing the β -galactosidase activity measured in larval extracts prepared at different times after natural infection from third instar larvae that express β -galactosidase under the control of the *Drs* promoter (*Drs*–*lacZ*). β -galactosidase activity in L-NAME-fed, infected larvae are consistently half of those in D-NAME-fed, infected larvae. The value for each time point is the average of three independent measurements. (H) Graph showing β -galactosidase activity in *Drs*–*lacZ* larvae prior to treatment with

SNAP and 24 h after treatment with SNAP. Provision of SNAP to uninfected larvae significantly increases β -galactosidase activity. Data are the average of three independent measurements.

treated *Dipt*–*lacZ* larvae, collected hemocytes 16 h later, and stained for β -galactosidase activity. We did not observe *Dipt*–*lacZ* activity in hemocytes from uninfected larvae (Fig. 5A), whereas a subset of hemocytes from larvae infected with *Ecc* had deposits of X-gal derived stain (Fig. 5B). This induction was also observed following treatment of uninfected larvae with SNAP (Fig. 5C). Infection-dependent induction of *Dipt* was blocked by L-NAME (Fig. 5D). We conclude that NO is necessary for efficient induction of *Dipt* in larval hemocytes following natural infection.

In mammalian systems, one of three NOS isoforms, inducible NOS (iNOS/NOS2), is up-regulated in macrophages in response to several types of infection or challenge with bacterial lipopolysaccharides. We asked whether infection up-regulates NOS in *Drosophila* hemocytes, the majority of which are phagocytic cells that function like mammalian macrophages. Larvae were infected by feeding with *Ecc*, and hemocytes were collected after 5 h and examined for NOS protein levels by immunofluorescence. We used two different anti-NOS antibodies, a universal anti-NOS and an antibody we prepared against a C-terminal peptide of *Drosophila* NOS. Similar results were observed for both antibodies. Hemocytes of infected larvae showed elevated NOS protein levels as early as 5 h after infection (Fig. 5H).

Because host pathogen interaction following natural infection is likely to first occur in the gut, we considered whether there might also be a response in this tissue. We monitored NOS activity in the guts of infected larvae using a histochemical stain (diaphorase staining). We observed a striking increase in NOS activity levels in the guts of naturally infected larvae 5 h after infection with *Ecc* (Fig. 5L).

NO-dependent activation of *Dipt* production in fat bodies requires the function of *domino*

Mutations in the chromatin-remodeling factor, *domino*, affect proliferating tissues, including imaginal tissues and larval hemocytes, and result in pupal lethality (Ruhf et al. 2001). It has been demonstrated previously that *domino* mutants are incapable of producing *Dipt* in their fat body after natural infection with *Ecc*, whereas fat body production of *Drs* was not impaired (Basset et al. 2000). This result has implicated hemocytes, which are missing in *domino* mutants, in the response to bacteria; however, as the mutation is relatively pleiotropic, other explanations for the *domino* dependence are possible. Nonetheless, we tested whether the *domino* mutation similarly impairs SNAP-mediated induction of *Drs*–GFP or *Dipt*–GFP.

Uninfected *Drs*–GFP larvae occasionally express GFP (Fig. 3F) and a low level of background expression persisted in *domino* mutants (Fig. 6A). As described previously, *Drs*–GFP; *domino* mutants expressed GFP after natural infection with *Ecc* (Fig. 6C). Interestingly, uninfected *domino* mutant larvae also induced *Drs*–GFP after feeding SNAP (Fig. 6D). Thus, *Drs* reporter induction in response to exposure to an NO donor or to bacteria can occur in the absence of hemocytes. Thus, cells other than, or in addition to, hemocytes must respond to NO and have inputs into the immune response.

We observed different effects when we examined *domino*; *Dipt*–GFP larvae. In this case, we observed no GFP fluorescence after either natural infection with *Ecc* or treatment with SNAP (Fig. 6F,G, respectively). These data show a parallel between NO signaling and the Imd pathway, in that both are blocked in the *domino* mutant.

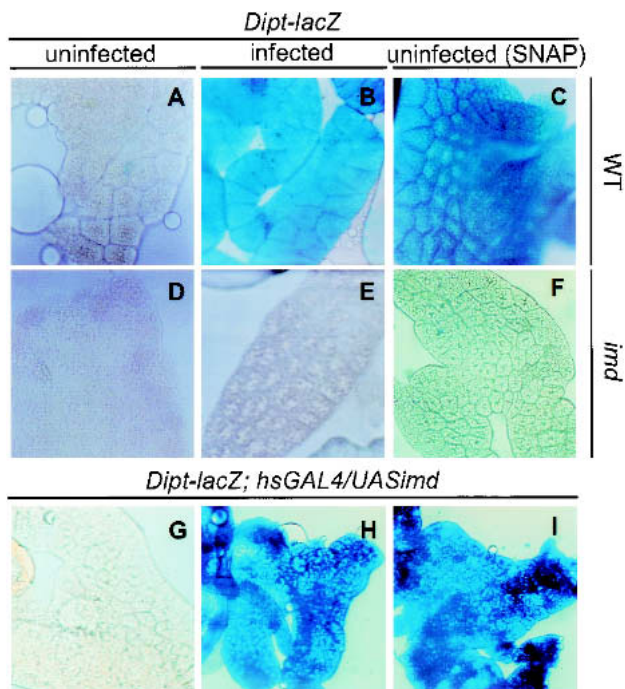


Figure 4. NO action in the fat body requires Imd. (A–I) Dissected fat bodies stained for β -galactosidase activity derived from a *Dipt-lacZ* reporter transgene. (A–C) In wild-type larvae carrying the *dipt-lacZ* transgene, natural infection (B) or exposure of uninfected larvae (C) to the NO donor SNAP induces *Dipt-lacZ* expression within 24 h (see Fig. 2H for measurement of induction levels). In contrast, no staining was observed in the fat bodies of *imd* mutant larvae 24 h after exposure to bacteria (E) or to SNAP (F). *hsGAL4* and *UAS-imd* transgenes allowed indirect induction of Imd expression in response to heat shock. The fat bodies of *dipt-lacZ*; *hsGAL4/UAS-imd* larvae showed no detectable *lacZ* staining in the absence of a heat shock (G), but staining was readily evident 24 h after administration of a heat shock, irrespective of whether the larvae had been larvae raised on D-NAME- (H) or L-NAME- (I) containing fly food.

Furthermore, this finding suggests that the fat body does not have an autonomous ability to respond to NO, and that the NO role in the induction of Dipt-GFP occurs at stages of signaling upstream of the fat body. Finally, the distinction between the effect of the domino mutation on Drs and Dipt reporters demonstrates that NO stimulates Drs expression via a pathway that is, at least partially, distinct from the pathway by which NO induces Dipt. We speculate that both pathways involve distinct sentinel tissues that act upstream of the fat body (Fig. 7).

Discussion

Drosophila has emerged as a potent model for elucidation of mechanisms underlying innate immunity. Our data show that NO performs a signaling function in *Drosophila* in the induction of host defenses to gram-negative bacteria. Pharmacological inactivation of *Drosophila* NOS makes larvae more susceptible to infection

and blocks Rel-dependent activation of the antimicrobial peptide Diptericin. NOS is up-regulated in the gut and hemocytes following natural infection, consistent with a role for NOS in combating invading micro-organisms. Furthermore, dietary provision of an NO donor is sufficient to activate promoters of antimicrobial genes in the hemocytes and fat body of uninfected hosts. Tests of the ability of NO to induce fat body expression of Diptericin in mutant backgrounds showed that it cannot bypass the requirement for Imd, a component of the signal transduction pathway inducing Dipt.

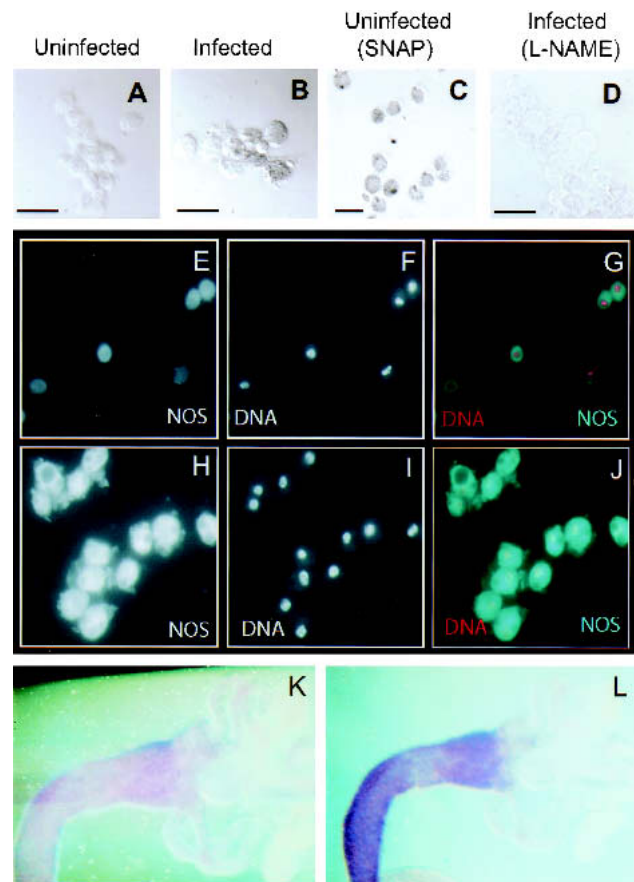


Figure 5. Expression of *Dipt-lacZ* in hemocytes requires NOS. (A–D) β -galactosidase activity in hemocytes from *Dipt-lacZ* larvae. Hemocytes from uninfected larvae have no β -galactosidase activity (A), whereas a subset of hemocytes from naturally infected larvae show staining (B). Provision of SNAP to uninfected larvae induces β -galactosidase (C), whereas L-NAME treatment of larvae inhibits induction upon natural infection (D). (E–J) Immunohistochemical staining showing NOS protein levels and Hoechst staining showing DNA in hemocytes from uninfected (E–G) and infected (H–J) larvae. E and F were merged in G, and H and I were merged in J with NOS in blue and DNA in red. The images in E and H were taken at the same magnification with identical exposure times. (K–L) The diaphorase staining of guts dissected from uninfected and naturally infected larvae. The diaphorase staining is significantly lower in the guts of uninfected larvae (K) compared with naturally infected larvae (L). Bar, 20 μ m.

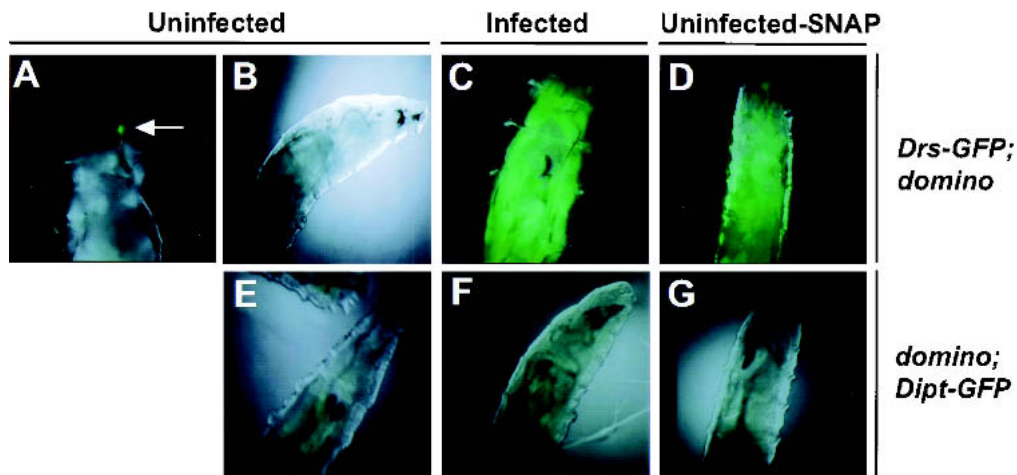


Figure 6. The *domino* mutant interferes with NO induction of *Dipt*, but not induction of *Drs*. (A–D) Expression of a *Drs-GFP* reporter transgene in the anterior of *domino* mutant third instar larvae. Localized GFP fluorescence is seen in a subset of uninfected larvae (A, arrow), whereas the majority of uninfected larvae do not exhibit detectable fluorescence (B). Natural infection (C) or provision of SNAP (D) to uninfected larvae induces the *Drs* reporter. (E–G) Fluorescence images of *domino* mutant third instar larvae carrying the *Dipt-GFP* transgene show that neither bacterial feeding (F) nor exposure to SNAP (G) induce expression of the *Dipt* reporter.

Inhibition of NOS impairs the host defense response

Our initial experiments showed that pharmacological inactivation of NOS compromised the ability of *Drosophila* larvae or adults to survive infection by septic injury or natural infection. Furthermore, NOS inhibition by L-NAME limited the ability of naturally infected larvae to eliminate gram-negative bacteria (*Ecc* and *E. coli*). L-NAME attenuation of pathogen elimination was particularly dramatic in larvae incapable of mounting Spz-dependent responses to infection. In contrast, an *imd* mutant was not further compromised by NOS inhibition. These results suggest that NOS activity is of particular importance in the Imd-dependent immune response pathway.

Production of *Dipt* in the fat body following infection relies entirely on the Imd-signaling cascade, as mutations in *imd* or the Imd-responsive NF- κ B homolog *rel* completely abrogate *Dipt* production following infection (Lemaitre et al. 1995a; Hedengren et al. 1999; Georgel et al. 2001). Overproduction of Imd in the absence of infection activates *Dipt* production (Georgel et al. 2001). Thus, the Imd pathway is required for *Dipt* activation and is sufficient to induce *Dipt* expression. Similarly, we show that pharmacological inhibition of NOS reduces *Dipt* expression, whereas introduction of NO donors activates *Dipt* expression. Thus, NO is required for optimal *Dipt* induction and NO is sufficient to trigger *Dipt* expression.

We propose that NO has a signaling role in the innate immune system of *Drosophila* and contributes to pathogen resistance in this capacity (Fig. 7). We note that this role in host defense is distinct from roles that NO might play as a cytotoxic agent used in the destruction of pathogens. Previous studies have indicated signaling functions for NO in the immune response of mammals and plants (Delledonne et al. 1998; Durner et al. 1998;

Diefenbach et al. 1999) although the exact manner in which NO functions has not been elucidated. Our data demonstrate that NO is essential for activation of a specific branch of the innate immune response pathway; the Imd pathway in *Drosophila* that is analogous to the TNF pathway of mammals.

Interactions between NO and the Tl-dependent response pathway

Our data imply a crucial role for NO in Imd-dependent signaling. However, NO also can activate production of Imd-independent antimicrobial peptides, as dietary provision of NO is sufficient to activate *Drs* production. Although this finding does not necessarily imply that NO normally acts in this pathway, it is of interest to consider the relationship to the results we have obtained for NO induction of the Imd pathway.

There is some cross-talk between the Toll and Imd pathways, such that activation of the Imd pathway by NO might contribute to the induction of *Drs*. However, the Imd pathway is not necessary for induction of *Drs* by exogenous NO, as a dietary NO source induces *Drs* to an equal extent in wild-type, *imd* and *rel^{E20}* mutants (data not shown). Hence, there must be another route of activation of *Drs* by exogenous NO. However, as inhibition of NOS does not effectively inhibit the Tl pathway as it does the Imd pathway, further analyses will be required to determine whether NO normally makes contributions to the Tl-dependent innate immune responses. Here, we focus on NO contributions to the Imd pathway.

The domino mutation and NO-dependent activation of antimicrobial peptide production

The *Domino* gene product is a transcription factor involved in chromatin remodeling (Ruhf et al. 2001). Mu-

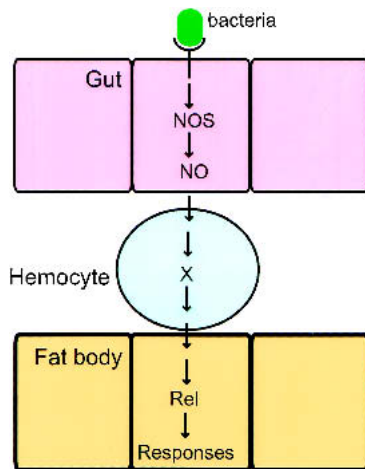


Figure 7. A model for NO signaling in immunity after natural infection of *Drosophila* larvae. Receptors, the PRRs on the surface of sentinel tissues, recognize molecules peculiar to pathogens. We suggest that the gut serves such a sentinel function in the case of natural infection, but note that other tissues such as hemocytes might adopt this role upon systemic infection. We propose that host-pathogen interactions up-regulate NOS in sentinel tissues (Fig. 5; Han et al. 2000) and activate a signaling cascade that leads to the release of a cytokine-like activity that recruits additional tissues to defend against the pathogen. We propose that hemocytes function as an intermediary in the Imd pathway that culminates in fat body production of antimicrobial peptides. It should be noted that the classical genes of the Imd pathway might act in different tissues or might act repeatedly at more than one tier of the cascade. As the septic injury model circumvents the gut as a point of entry for pathogens, it is likely that other tissues will act as sentinels mediating the response or the fat body might respond directly under these circumstances.

tations in *domino* affect proliferating tissues such as the imaginal discs and lymph glands, resulting in larvae with several defects, including elimination or severe reduction in the number of hemocytes (Braun et al. 1998). Whereas *domino* mutants produce Dipt in the proventriculus after natural infection, they no longer produce it in the fat body. These observations led to the proposal that hemocytes may produce a cytokine-like molecule following natural infection that activates antimicrobial peptide production in the fat body (Basset et al. 2000). Whereas the proposal that the fat body response is secondary to a hemocyte signal remains tentative because of the pleiotropy of the *domino* mutation, it is clear that a gut (proventriculus) response can occur independent of hemocytes. We find that NO also fails to induce Dipt in the fat body of *domino* mutant larvae, suggesting that NO does not act directly on the fat body. Perhaps it mediates signaling at earlier steps in the response.

We propose that different host organs play distinct roles in the response to foreign organisms. Tissues that contact a pathogen early during infection are well positioned to perform a sentinel function by detecting the pathogen and signaling other tissues to recruit their contributions to host defense (Fig. 7). Our results are consis-

tent with the suggested involvement of hemocytes in the response of the fat body (Basset et al. 2000). The finding that exogenous NO is unable to induce Dipt in the fat body of *domino* mutants, which lack hemocytes, suggests that the fat body cannot respond autonomously to NO. Although the pleiotropy of the mutation allows other interpretations, the result encourages us to think that NO acts upstream of the fat body. In support of this, the initial fat body response to NO or infection appears as a mosaic of Dipt expressing and nonexpressing cells. This feature is not easily consistent with a humoral signal, but is compatible with an induction by cell contact with activated hemocytes. Furthermore, hemocytes respond to natural infection by inducing Dipt. Because this response is mimicked by NO treatment and inhibited by pharmacological inhibition of NOS, we suggest that NO acts upstream of this hemocyte response. It was shown previously that the gut (proventriculus) can respond to infection independent of, and perhaps upstream of, hemocytes (Basset et al. 2000). We show that NOS activity is induced in the gut following natural infection. These findings suggest the tentative model shown in Figure 7, in which cells in the gut detect the pathogen, activate hemocytes via an NO dependent signal, and hemocytes act in turn to induce the fat body by an as yet unknown signal. As pathogens have numerous ports of access, we expect that, like the diverse dendritic system in humans, there will be a number of alternative sentinel tissues and multiple pathways of triggering activation of innate immunity.

Our work and other studies indicate that NO has a global involvement in the immune response. Work in mammalian cells predicted involvement of NO in pathogen elimination during an oxidative burst in phagocytic cells, whereas our work documents a role for NO as an inducer of the innate immune response. It remains to be tested fully, but several observations suggest that both of these roles will be conserved.

Materials and methods

Fly strains and culturing

We used Sevelen flies as a wild-type strain. The *spz^{rm7}*, *domino*, and *imd* mutant strains used have been described elsewhere (Lemaitre et al. 1995a, 1996; Braun et al. 1998; Georgel et al. 2001). The reporter strains *Dipt-lacZ*, *Drs-GFP*, and *Drs-lacZ* have been described in Reichhart et al. (1992), Ferrandon et al. (1998), and Manfrulli et al. (1999), respectively. *Dipt-GFP* flies were a gift of Bruno Lemaitre. It is a homozygous viable strain with two insertions on each third chromosome. In this line, GFP is expressed under the control of the *Dipt* 2.2-kb promoter, which recapitulates in vivo *Dipt* expression. Flies were cultured on standard fly food at 25°C prior to infection. After infection, flies were transferred to a 29°C incubator.

Infection experiments

Natural infection experiments with *Ecc* or *E. coli* were done as described previously (Basset et al. 2000). Briefly, for 30 min, third instar larvae were fed a concentrated bacterial pellet

mixed into crushed banana with 500 mM L-NAME or D-NAME. Larvae were then transferred to 29°C for the remainder of the experiment. To determine the consequences of NOS inhibition on larval survival after infection, larvae were transferred to vials that contained instant fly food with 100 mM L-NAME or D-NAME, respectively. Bacterial titer was determined by plating serial dilutions of homogenate from infected larvae at various times after infection onto LB-Amp plates. The strain of *Ecc* used (015) is ampicillin resistant and *E. coli* was transfected with a β -lactamase encoding plasmid. For septic injury experiments on larvae, late second instar larvae were transferred from standard fly food to instant fly food containing 100 mM D-NAME or L-NAME. A total of 120 third instar larvae were picked 16 h later and infected by puncturing the cuticle with a dissecting needle that had been soaked previously in a concentrated bacterial pellet. In parallel control experiments, 120 larvae were treated in an identical manner and injured with a sterile needle. For septic injury of adults, late second instar larvae were transferred from standard cornmeal food to instant fly food with 100 mM L-NAME or D-NAME. Two-hundred 1 to 2-day-old adult flies that eclosed were infected by puncturing their thorax with a sharp needle that had been dipped in a concentrated bacterial pellet.

Microscopy

All images were taken on a Leica DMRD. To visualize expression of GFP in larvae, darkfield and green fluorescent images (FITC) were taken of representative larvae and merged using Adobe Photoshop 5.5. Figures were assembled using Adobe Illustrator. For immunohistochemistry of larval hemocytes, hemocytes were deposited on Superfrost Plus Gold microscope slides (Fisher) and fixed for 5 min in 4% glutaraldehyde in PBS. Cells were incubated with a 1:200 dilution of Rabbit-anti-NOS and Rhodamine-labeled anti-rabbit was used a secondary antibody. NOS antibody staining was done using a universal anti-NOS antibody (Oncogene) or using an antibody raised against a peptide (TAEIHTKSRATARIRMASQ) that corresponds to a C-terminal section of the mature NOS protein.

β -Galactosidase assays

To monitor induction of *Dipt-lacZ* in fat bodies, fat bodies were dissected from larvae in PBS and fixed for 10 min in PBS with 0.5% glutaraldehyde on ice. Fat bodies were then incubated at room temperature in staining buffer [30 μ L X-gal (5% 5-bromo-4-chloro-3-indolyl β -D-galactosidase in DMFO) per milliliter staining solution (10 mM of sodium phosphate buffer at pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 3.5 mM K₃FeCN₆, and 3.5 mM K₄FeCN₆)]. β -galactosidase activity in hemocytes from *Dipt-lacZ* larvae was monitored by depositing hemolymph on Superfrost Plus Gold microscope slide (Fisher), fixing for 30 sec in PBS with 0.5% glutaraldehyde, and staining overnight at 37°C with staining buffer. β -galactosidase titration was done as described previously (Basset et al. 2000).

Diaphorase staining

Third instar larvae were washed and dissected in PBS and then transferred to fixing buffer (4% paraformaldehyde, 100 mM PIPES at pH 7.4, 2 mM MgSO₄, 1 mM EGTA) for 30 min at room temperature. Larvae were then washed in PBS and incubated in staining solution (1 mM NADPH, 0.2 mM nitrobluetetrazolium, 100 mM Tris at pH 7.2, 0.2% Triton X-100) for 20 min at room temperature. Larvae were washed in PBS and mounted in glycerol.

Pharmacology

The NO donor SNAP was fed to third instar larvae for 15 min at room temperature by mixing 15 mM SNAP in crushed banana and then feeding to larvae.

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