Attenuation of host NO production by MAMPs potentiates development of the host in the squid–vibrio symbiosis

Melissa A. Altura,1 Eric Stabb,2 William Goldman,3 Michael Apicella4 and Margaret J. McFall-Ngai1*
1Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI, USA.  
2Department of Microbiology, University of Georgia, Athens, GA, USA.  
3Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA.  
4Department of Microbiology, University of Iowa, Iowa City, IA, USA.

Summary

Bacterial pathogens typically upregulate the host’s production of nitric oxide synthase (NOS) and nitric oxide (NO) as antimicrobial agents, a response that is often mediated by microbe-associated molecular patterns (MAMPs) of the pathogen. In contrast, previous studies of the beneficial Euprymna scolopes/Vibrio fischeri symbiosis demonstrated that symbiont colonization results in attenuation of host NOS/NO, which occurs in high levels in hatching light organs. Here, we sought to determine whether V. fischeri MAMPs, specifically lipopolysaccharide (LPS) and the peptidoglycan derivative tracheal cytotoxin (TCT), attenuate NOS/NO, and whether this activity mediates the MAMPs-induced light organ morphogenesis. Using confocal microscopy, we characterized levels of NOS with immunocytochemistry and NO with a NO-specific fluorochrome. When added exogenously to seawater containing hatching animals, V. fischeri LPS and TCT together, but not individually, induced normal NOS/NO attenuation. Further, V. fischeri mutants defective in TCT release did not. Experiments with NOS inhibitors and NO donors provided evidence that NO mediates apoptosis and morphogenesis associated with symbiont colonization. Attenuation of NOS/NO by LPS and TCT in the squid–vibrio symbiosis provides another example of how the host’s response to MAMPs depends on the context. These data also provide a mechanism by which symbiont MAMPs regulate host development.

Introduction

Nitric oxide (NO) is involved in a wide array of biological processes in animals, including cell signalling, neurotransmission and innate immunity. As an easily diffusible gas, NO acts as an intra- and intercellular signalling molecule (reviewed in Martinez-Ruiz and Lamas, 2009). As a reactive nitrogen species, NO is known to be important in host responses to bacterial pathogens, particularly as a bactericidal and bacteriostatic compound (Zhu et al., 1992; Fang, 1997; Miller and Britigan, 1997; Bolwell, 1999; Flak and Goldman, 1999; Chan et al., 2001; Brennan et al., 2004; Deupree and Schoenfisch, 2009). The role of NO has long been studied in pathogenesis and is now being explored in beneficial associations. In diseases where symbiotic associations are perturbed, such as inflammatory bowel disease and Crohn’s disease, NO levels are elevated and play a role in inflammation (Kimura et al., 1997). In the legume-rhizobium symbiosis, NO levels in the plant root are initially high upon contact with the symbiont and then drop during colonization (Herouart et al., 2002; Nagata et al., 2008).

The squid–vibrio symbiosis is a model for studying beneficial animal–bacterial interactions (reviewed in Nyholm and McFall-Ngai, 2004; Visick and Ruby, 2006). The role of NO has been studied in this system from the perspective of both partners (Davidson et al., 2004; Wang et al., 2010a,b). The nocturnal Hawaiian bobtail squid, Euprymna scolopes (Fig. 1A), apparently uses the luminous Gram-negative bacterium, Vibrio fischeri, as part of an anti-predator strategy (Jones and Nishiguchi, 2004; reviewed in Stabb and Millikan, 2009). The bacteria are horizontally transmitted i.e. the juvenile squid must harvest the bacteria from the surrounding seawater anew each generation rather than acquiring the symbiont via maternal transmission. Once the squid has been colonized by its symbiont, it maintains the bacteria in a
specialized set of tissues known as the light organ, a bi-lobed structure that includes the ink sac and associated tissues (Fig. 1B). Each lateral surface of the juvenile light organ is covered with a ciliated epithelium that includes a set of appendages, which extend from the surface of the light organ into the mantle cavity. These appendages aid in the circulation of water over the surface of the light organ and shed mucus in which the initial colonizing population of symbionts are harvested. At the base of the larger, anterior appendage are three pores, each of which leads though a duct to an antechamber and finally to a crypt, where the bacteria reside for the life of the animal (Fig. 1C).

The process of obtaining and cultivating these bacteria leads to a series of physiological and biochemical changes to the light organ, including the remodelling of the surface epithelium and the attenuation of nitric oxide production (Davidson et al., 2004; Koropatnick et al., 2004). After the squid has been infected by the symbiotic V. fischeri from the seawater, the ciliated epithelium, including the appendages, is shed as part of the development into the adult form of the light organ (Montgomery and McFall-Ngai, 1994). This morphogenic process is gradual, occurring over the first 4 days following colonization. It is apoptotic and induced largely by cell-envelope products, or MAMPs (microbe-associated molecular patterns) from V. fischeri, which are presented by the symbionts to sensitive epithelia inside the light organ (Foster and McFall-Ngai, 1998; Foster et al., 2000; Koropatnick et al., 2004). Specifically, early-stage apoptosis (chromatin condensation), which is induced by the lipid A portion of lipopolysaccharide (LPS), begins around 6–12 h post infection. Late-stage apoptosis, which is characterized by DNA fragmentation, begins 12–14 h post exposure and continues until the epithelium is completely regressed. This later process is induced synergistically by the peptidoglycan (PGN) monomer tracheal cytotoxin (TCT) and lipid A (Foster and McFall-Ngai, 1998; Foster et al., 2000; Koropatnick et al., 2004; Troll et al., 2009). The process by which symbiosis-associated apoptosis occurs in the squid is poorly understood, although p53 and a host PGN-recognition protein do appear to play a role (Goodson et al., 2006; Troll et al., 2009).

Previous work on the squid–vibrio system demonstrated that NO and NOS are regulated in response to symbionts. In addition to being present in the ciliated appendages, NO and NOS levels are relatively high in the ducts and antechambers of the light organs of newly hatched animals. During the first 18 h after hatching, aposymbiotic (APO) animals maintain a high level of NOS/NO, while symbiotic (SYM) animals have lower levels of NOS/NO in and on the surface of their light organs (Davidson et al., 2004). However, the mechanisms regulating NOS/NO attenuation have remained unexplored.

As MAMPs are implicated in the control of both pathogenic and beneficial symbioses, the present study was undertaken to examine the role of bacterial products, specifically LPS and PGN derivatives, in regulating NOS/NO levels in the early stages of the squid–vibrio symbiosis. In addition, as NO can function in the control of apoptosis in animal cells, we sought to determine whether symbiosis-induced NO attenuation plays a role in the symbiosis-
induced apoptosis and the eventual morphogenesis that are hallmarks of the developmental program of the light organ. These data provide a link between the MAMP-induced developmental program and symbiont-induced NOS/NO attenuation in this system.

Results

LPS and TCT work synergistically to attenuate NOS/NO in the light organ

To determine whether V. fischeri MAMPs are active in the NOS/NO attenuation process, we exposed juvenile E. scolopes to LPS, TCT, or both. Animals exposed to both LPS and TCT had levels of universal NOS antibody (uNOS) staining that were attenuated by approximately 3.5-fold and were statistically different from APO animals and statistically indistinguishable from SYM (Fig. 2). Concentrations of LPS and TCT that were determined from previous studies to induce symbiosis-associated phenotypes in the animal (Foster et al., 2000; Koropatnick et al., 2004) were also active for NOS/NO attenuation (Fig. 2C). In contrast to animals exposed to both LPS and TCT, animals exposed to either LPS or TCT alone had levels of NOS that were statistically indistinguishable from APO animals (Fig. 2). We observed no statistically significant changes between the different treatments in nerve bundles labelled with the uNOS antibody, which were used as internal controls (Fig. S1). Control APO animals had statistically significantly higher levels of uNOS antibody labelling in the cells lining their ducts and antechambers as compared with control SYM animals at 18 h post exposure to wild-type V. fischeri. These findings are consistent with previous data (Davidson et al., 2004).

To determine whether the changes in NOS protein levels were correlated with changes in NO levels, we exposed animals to the same treatments as in the previous experiment, and then stained them with DAF-FM (4-aminomethylfluorescein) an indicator of NO. The changes in NO levels under the different conditions correlated with changes in uNOS antibody labelling. High levels of NO production in the light organ characteristic of APO animals were attenuated by the onset of colonization with V. fischeri (SYM). Animals exposed to both LPS and TCT together exhibited a SYM-like pattern, i.e. more attenuated, punctate staining, whereas animals treated with solely LPS or TCT had a bright staining pattern like that of APO animals (Fig. 3).

The effects of altered MAMP presentation and MAMP specificity

Juvenile squid were exposed to a V. fischeri strain defective in TCT release (ltg) to further test whether NOS attenuation was caused by the synergistic activity of LPS and TCT. The ltg strain releases levels of TCT below the limit of detection (Adin et al., 2009). Squid exposed to ltg bacterium had NOS labelling of light organ tissues statistically indistinguishable from APO animals. Complementation of TCT release either pharmacologically (ltg + TCT) or genetically, specifically with one of the lytic transglycosylases on a multicopy plasmid (ltg + ltgA and ltg + ltgD), restored NOS attenuation to levels statistically different from APO animals and indistinguishable from SYM animals. Bacteria with the vector plasmid used for genetic complementation without either of the ltg genes (ltg + empty vector) were unable to induce NOS attenuation and were statistically indistinguishable from APO (Fig. 4A).

To determine the specificity of the host response to LPS, we exposed juvenile squid to TCT and LPS isolated from non-symbiotic bacteria, such as Neisseria gonorrhoeae, Neisseria meningitidis and Haemophilus influenzae. We used these strains because their LPS structure has been characterized and because they are known to elicit changes in NOS/NO levels in pathogenic systems. We found that TCT and LPS from non-V. fischeri bacterial cells were able to induce attenuation of NOS to levels that were different from APO animals but indistinguishable from SYM animals (Fig. 4B). These data provide evidence that, similar to MAMPs induced morphogenesis, the NOS attenuation response is not specific to V. fischeri LPS (Foster et al., 2000).

NO plays a role in the developmental program of the squid

Lipopolysaccharide and TCT have previously been shown to induce symbiosis-dependent apoptosis of the light organ epithelium (Foster et al., 2000; Koropatnick et al., 2004). To determine whether the process of MAMP-induced NO attenuation and apoptosis are linked, we first exposed squid to an NOS inhibitor, S-methyl-thiocitrulline (SMTC), and measured its effects on both early- and late-stage apoptosis. We found that, at concentrations where an appreciable change in DAF staining could be observed (i.e. 100 μM) (Fig. S2A and B), SMTC induced an increase in the labelling of nuclei for early-stage apoptosis in the light organ epithelium of both APO and SYM animals that was statistically different from APO untreated animals, but statistically indistinguishable from SYM untreated animals (Fig. 5A and B). However, SMTC was unable to induce late-stage apoptosis in APO animals (Fig. 5B). In addition to finding no increase in late-stage apoptosis in APO animals in the presence of a NOS inhibitor, we also found no significant progression through the stages of regression, which result from apoptosis in wild-type infections. However, we did observe accelerated
Fig. 2. The visualization and quantification of NOS attenuation in the presence and absence of *V. fischeri* MAMPs by LSM confocal microscopy.

A. Representative micrographs of duct cells labelled with a uNOS antibody and a FITC-conjugated secondary antibody (green). The left column depicts duct tissues from representative animals. The orange box indicates the field shown in the right hand column, which is a magnified view of individual duct cells. Counterstain: Actin, rhodamine phalloidin (red) and nuclei, TOTO-3 (blue). Bars, 10 µm.

B. The quantification (see Experimental procedures) of a single representative microscopy experiment in which animals were exposed to different MAMPs (aposymbiotic *n* = 10, symbiotic *n* = 12, LPS *n* = 8, TCT *n* = 9, LPS + TCT *n* = 14).

C. The quantification of uNOS fluorescence in antechamber cells of a single representative microscopy experiment in which the animals were exposed to varying concentrations of *V. fischeri* LPS and 1 µM TCT. Bars, standard error. The asterisks '*' indicate data points that were significantly different from symbiotic conditions.
regression of the ciliated epithelium in SYM animals that were exposed to NOS inhibitor as compared with unexposed SYM controls (Fig. 5C). We also found that late-stage apoptosis increased with the addition of either LPS or TCT and the NOS inhibitor as compared with LPS or TCT untreated controls. LPS and TCT typically do not induce late-stage apoptosis individually. Animals treated with both LPS and TCT in the presence or absence of inhibitor were indistinguishable from each other (Fig. 5D). Typically, both LPS and TCT are required to induce late-stage apoptosis. We confirmed a decrease in NO levels in target tissues with DAF-FM staining, visualized by LSM-confocal microscopy. To determine whether the effects of the inhibitor are specific to the light organ and cells producing NO, we analysed other squid tissues. We did not observe the induction of non-specific apoptosis in other tissues (data not shown).

We also treated squid with an NO donor, S-nitroso-\(N\)-acetylpenicillamine (SNAP). We found that SNAP, at effective working concentrations (Fig. S2A and C), provided protection against symbiosis-dependent apoptosis in the light organ epithelium. SYM animals treated with SNAP showed no detectable or low levels of early- and late-stage apoptotic cells in the light organ epithelium that were statistically indistinguishable from untreated and treated APO animals. Untreated SYM animals showed high levels of apoptosis that were statistically different from the other treatments (Fig. 5A and B). We confirmed by confocal microscopy the presence of increased NO in target tissues of treated animals (Fig. S2A). However, we did not observe the induction of apoptosis in adjacent control tissues (data not shown).

**Discussion**

Our findings of NOS/NO attenuation by bacterial MAMPs and its involvement in normal bacterially induced developmental processes increase the scope of biologists’ views of the relationship between host-derived nitrosative compounds and their impact on bacterial colonization. Our specific findings provide evidence that in the squid–vibrio symbiosis: (i) NOS and NO production are regulated by the synergistic activity of the symbiont MAMPs, LPS and TCT; (ii) mutants deficient in TCT release are defective in normal NOS reduction; (iii) NOS reduction is not the sole inducer.

Studies with MAMPs, individually and in combination, as well as NO inhibitors and NO donors, have provided insight into the underlying mechanisms of bacterially induced host tissue development. The data resulting from this study provide evidence that NOS attenuation is necessary but insufficient to trigger late-stage apoptosis. Also, these data suggest that LPS and TCT are able to induce late-stage apoptosis individually in the presence of the NOS inhibitor, whereas under natural conditions,
Fig. 5. The role of NO in the induction of apoptosis.
A. Representative micrographs of the anterior appendages of light organs stained with either acridine orange (Top row) or TUNEL (green) and rhodamine phalloidin (red) (bottom row). Arrows indicate apoptotic nuclei.
B. Quantification of single representative experiments of animals exposed to NO donor (SNAP) or inhibitor (SMTC) as indicated and quantified for either early-stage (acridine orange) or late-stage (TUNEL) apoptosis. The number of apoptotic nuclei was counted per appendage for each treatment.
C. Graph of single representative experiment of animals exposed to NOS inhibitor (SMTC) and scored for stage of regression.
D. Graphs of average of two experiments of animals exposed to NOS inhibitor (SMTC) and/or bacterial MAMPs as indicated and quantified for late-stage (TUNEL) apoptosis. The number of apoptotic nuclei was counted per appendage for each treatment. Statistical analyses were done comparing the samples indicated by brackets using a Student's T-test with a Bonferroni adjustment. The asterisk '*' indicates significance of $P \leq 0.05$. n.s., not significantly different.
these MAMPs must both be present to allow late-stage apoptosis to proceed. Taken together, these findings indicate that these MAMPs act on the apoptosis pathway at a minimum of two points in the process, and support a model of MAMPs/NO activity in host light-organ morphogenesis (Fig. 6). In this model, the synergistic activity of LPS and TCT is required for NO attenuation to occur, which would induce the host epithelial cells to enter early-stage apoptosis. Once NO levels are attenuated, the system is permissive to entry into late-stage apoptosis, and eventual morphogenesis, through induction by either LPS or TCT. Thus, NO attenuation removes a block on apoptosis and, concomitantly, LPS or TCT activates other pathways to allow apoptosis to proceed. For example, an earlier study of symbiont-induced apoptosis in the squid host demonstrated the involvement of the p53 protein (Goodson et al., 2006), which could be the NO-independent pathway suggested in the second stage of the model. Alternatively, such a biphasic activation of apoptosis may be controlled by the receptor–ligand interactions of the MAMPs and host pattern recognition receptors (PRRs), which are abundant in light organ tissues and known to be involved in apoptosis (Goodson et al., 2006; Troll et al., 2009).

The bacterially induced decrease in NO production by LPS and TCT in the squid–vibrio system is the inverse of what is known to occur in pathogenic interactions. LPS is a well-documented inducer of nitric oxide production in innate immune cells (Hauschmidt et al., 1990; Costa et al., 2009), and TCT is also known to induce NO production in animal tissue models (Flak and Goldman, 1999). A synergy of MAMPs in evoking a host response, demonstrated with both pharmacological exposure to MAMPs and genetic manipulations of V. fischeri MAMP production, is also a common feature of response to bacterial products in pathogenesis. LPS acts synergistically with other inflammatory bacterial products, such as bacterial DNA and TCT (Gao et al., 1999; Flak et al., 2000), and host cytokines, such as interferon-gamma and IL-1 (Xie et al., 1992; Geller et al., 1993) to regulate NO production. Most regulation of NO by bacterial products and cytokines is thought to be transcriptional (Lowenstein et al., 1993; Xie et al., 1993; Kunz et al., 1994; Ganster et al., 2001; Blanchette et al., 2009); however, NO can also be regulated by protein and message stability (Vodovotz et al., 1993; Geng and Lotz, 1995; Musial and Eissa, 2001), substrate availability (Chaturvedi et al., 2007), and protein–protein interactions, including interactions with the cytoskeleton (Zimmermann et al., 2002; Su et al., 2003). Based on the gradual nature of NO attenuation in the squid–vibrio system, occurring over a 12 h period, and the strong correlation between NO and NO levels, it is possible that NO is regulated transcriptionally. However, several NO-regulating proteins, NOSTRIN and NOSIP, have been found in the light organ EST database (Chun et al., 2009), and NO attenuation temporally coincides with perturbations to the cytoskeleton within the light organ (Kimbell and McFall-Ngai, 2004). In addition, the light organ has a diverse set of pattern-recognition receptors, including PGN recognition proteins and LPS-binding proteins (Goodson et al., 2005), and the genes encoding these factors are differentially regulated in response to the presence of symbionts (Chun et al., 2008). At present, we are examining these receptors as candidate molecules in the synergistic regulation of NO, apoptosis and morphogenesis (Goodson et al., 2005; Troll et al., 2009).

The NO attenuation in the presence of LPS from several different species indicates that attenuation is not specific to V. fischeri LPS. These data are consistent with other work from the squid–vibrio system, which indicates that LPS from Vibrio cholera, Escherichia coli and H. influenzae are able to induce apoptosis as measured by fluorescence staining of chromatin-condensed nuclei (Foster et al., 2000). Additionally, V. fischeri has mechanisms for sensing and detoxifying NO, which may aid in its ability to bypass the nitrosative stress of the light organ and deliver their MAMPs to the host more effectively (Wang et al., 2010a,b). It is important to note that only V. fischeri is capable of entering the deep crypts where the signals for host morphogenesis are received. As such, purified MAMPs from non-symbiotic bacteria can be delivered to the crypts at sufficient concentrations to induce host responses; however, under natural conditions the animal would not see sufficient LPS and TCT levels within the crypts to induce host morphogenesis in the absence of V. fischeri colonization. Additionally, the timing of NO attenuation, which is initiated at 6 h post exposure, occurs only after specific colonization by V. fischeri has been established. Thus, the induction of development...
by MAMPs likely depends on the presentation of these molecules by \textit{V. fischeri} at the right time and in the right place within the light organ.

Pharmacological inhibition of NO induced early-, but not late-stage apoptosis, whereas artificial supplementation of NO levels protected against apoptosis of the ciliated epithelium. These findings suggest that NO attenuation is required for the induction of apoptosis, but is not sufficient for it to proceed to completion. Work in other mollusks has demonstrated a role for NO in development. In \textit{Ilyanassa obsolete}, a mud snail, the apical ganglion (AG) is lost during metamorphosis (Lin and Leise, 1996). The apoptotic process is induced by decreased NO levels in the AG and can be prevented with the addition of exogenous NO (Leise et al., 2004; Gifondorwa and Leise, 2006). The apoptotic process is typically a multistep, multifactorial process and can vary from tissue to tissue. For analysis of the squid–vibrio system, we have identified host characters that are specific to both the early (e.g. chromatin condensation) and late (e.g. DNA breakdown by endonuclease activity) stages of apoptosis, and found that different combinations of symbiont products induce them. Here we demonstrate a role for high NO levels in the prevention of apoptosis. However, due to the inability of this NOS inhibitor to induce late-stage apoptosis and full regression, it seems likely that the induction of apoptosis in the ciliated epithelium is a complex multistage process, requiring additional yet unidentified signals for the entire program to progress. Future studies of the system will be aimed at unravelling the complex interplay between these various elements.

In summary, our data show that LPS and TCT induce NOS/NO attenuation in the squid–vibrio system and provides evidence for the role of NO in the symbiosis-induced development of the squid host. These findings further support the importance of context in determining the host response to MAMPs based on whether the partners are in a beneficial or pathogenic interaction. In addition, our work expands the role of NO in host–microbe interactions beyond that of antimicrobial compound and examines its role as a host-signalling molecule within these systems.

**Experimental procedures**

**General methods**

Adult \textit{E. scolopes} were caught off the coast of Oahu and bred in salt water tanks as previously described (Montgomery and McFall-Ngai, 1993). Juveniles obtained from this breeding colony were collected within 15 min of hatching and washed three times in instant ocean that had been passed through a 0.45 \(\mu\)m filter (FSIO) to remove any trace amounts of bacteria and their products. Animals were maintained on a 12 h light/dark cycle during the course of the experiment. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted, and all confocal experiments were performed on a Zeiss 510 laser scanning confocal microscope.

We used juvenile \textit{E. scolopes} with (SYM) and without (APO) the symbiotic \textit{V. fischeri}, as well as juveniles colonized with specific \textit{V. fischeri} mutants. APO juveniles were maintained in FSIO free of \textit{V. fischeri} over the course of the experiment. SYM animals and those exposed to mutant strains of \textit{V. fischeri} were maintained in FSIO with 5000 colony forming units ml\(^{-1}\) of wild-type (WT) \textit{V. fischeri} strain ES114 or the indicated mutant strain overnight. Strains of \textit{V. fischeri} that are defective in TCT release consisted of a mutant ltg\(^{-}\) (ES114-derivative strain DMA388: \textit{ltgA}, \textit{ltgD}, \textit{ltgY::erm}) and its complemented strains (ltg\(^{-}\) + \textit{ltgA}, ltg\(^{-}\) + \textit{ltgD}, ltg\(^{-}\) + empty vector) (Adin et al., 2009; Troll et al. 2009). Animals exposed to purified LPS from non-symbiotic bacteria were also exposed to 1 \(\mu\)M TCT simultaneously. Animals exposed to purified bacterial surface components were given a single dose at the beginning of the experiment and maintained in FSIO with 10 ng ml\(^{-1}\) of LPS derived from \textit{V. fischeri} or other species and/or 1 \(\mu\)M TCT in non-tissue culture-treated 24-well plates for the length of the experiment (18–20 h). We also confirmed that these concentrations of MAMPs, which were determined to be effective for the induction of light organ morphogenesis, are also optimal for the induction of NOS/NO attenuation (Fig. 2C). Colonization of the animals was monitored by taking luminescence readings using a TD 20/20 luminometer and animals that were improperly colonized were eliminated from the study. Bacterial LPS and TCT were isolated using previously described methods (Cookson et al., 1989; Apicella et al., 1994; Apicella, 2008).

**The detection of NO by DAF-FM staining**

We examined NO production in APO and SYM animals, as well as in APO animals exposed to purified bacterial products. DAF-FM (Invitrogen, Carlsbad, CA, USA) was used to detect NO production in the squid light organ as previously described (Davidson et al., 2004). Juvenile squid were exposed to conditions of APO, SYM, or bacterial products for 18–20 h, which was previously described as the time by which complete symbiotic NO attenuation could be observed. The animals were then incubated in 5 \(\mu\)M DAF-FM for 30 min, washed for 1 min in FSIO, anesthetized in 2\% ethanol in FSIO, and ventrally dissected on a depression slide. Each animal was then viewed with a confocal microscope.

**The detection of NOS by immunocytochemistry**

A universal NOS antibody (uNOS) (Thermo Fisher Scientific, Rockford, IL, USA) was used to detect NOS in the squid light organ. Juvenile squid were anesthetized in 2\% ethanol in FSIO and then fixed overnight in 4\% paraformaldehyde in marine phosphate buffered saline (mPBS) (50 mM sodium phosphate, 0.45 M sodium chloride, pH 7.4) at 4\(^\circ\)C. Light organs were removed from the squid and permeabilized for 48 h in 1\% Triton X-100 in mPBS. Then, the light organs were blocked overnight at 4\(^\circ\)C in blocking solution (mPBS with 0.5\% bovine serum albumin, BSA, 1\% goat serum, and 1\% Triton X-100). The light organs were then incubated with the uNOS antibody at a dilution of 1:50 in blocking solution for 7 days at 4\(^\circ\)C. Samples were rinsed 4 \(\times\) 1 h in 1\% Triton X-100 in mPBS and incubated overnight in blocking solu-
tion at 4°C. Samples were then incubated with a 1:50 dilution of fluorescein-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in blocking solution in the dark at 4°C overnight. To counterstain the actin cytoskeleton, the samples were rinsed in 4 × 30 min in 1% Triton X-100 in mPBS, and then incubated overnight with rhodamine phalloidin in mPBS with 0.5% Triton X-100 in the dark at 4°C. The light organs were then washed 2 × 10 min with 0.5% Triton X-100 in mPBS followed by 2 × 10 min in mPBS. To counterstain the nuclei, the light organs were washed 3 × 10 min in 2× SSC (30 mM sodium citrate, 300 mM sodium chloride, pH 7.2) and then incubated in 2× SSC with 100 ng ml⁻¹ RNase A for 30 min at 37°C. The samples were washed 3 × 1 min in 2× SSC and then incubated for 20 min with TOTO-3 in 2× SSC at room temperature. The samples were washed 3 × 1 min in 2× SSC and then 2 × 5 min in mPBS. Samples were mounted on glass slides in VectaShield (Vector Laboratories) to prevent photo-bleaching and then examined by confocal microscopy.

Quantification of uNOS staining

Mean fluorescence of the cytoplasmic portion of the duct and antechamber cells was measured using the LSM 510 confocal software. Background fluorescence was subtracted from these values. The background-subtracted values were then normalized against the staining of the nerve bundles, an internal control whose fluorescence was unaffected by the presence or absence of symbiosis. The resulting values were subjected to an ANOVA analysis followed by a Tukey’s pairwise comparison to determine the significance of the difference between individual samples at a P-value of < 0.05.

Exposure of squid to NO donors and inhibitors and associated cell death assays and regression assays

Squid exposed to NO-modulating chemicals were kept in scintillation vials filled with the relevant chemical and FSIO overnight. Squid were exposed every 5 h to the NO donor S-methyl-N-acetyl-penicillamine (SNAP), which has a half-life of 6 h at 25°C, at a concentration of 10 μM for a total of 18 h. To determine the amount of SNAP to use, we exposed the squid to several concentrations and found the concentration that did not appreciably affect the animals’ behaviour or survival, but still elicited a level of NO, measured by DAF-FM staining, in SYM animals that was comparable to SYM unexposed controls (Fig. S2A and B). We also performed growth curve experiments on V. fischeri to make sure that the inhibitor and donor did not affect the bacterial growth rate. The bacteria were unaffected by the inhibitor, while the donor-exposed bacteria showed a brief lag phase and then caught up to unexposed controls (data not shown). Following incubation, the animals were stained with DAF-FM as previously described to determine the effect of these chemicals on NO levels.

To assess the progression of cell death in the superficial epithelium of the light organ, a cohort of animals was stained with acridine orange, which allows visualization of early-stage apoptosis, and another was fixed as previously described and stained with TUNEL, which allows visualization of late-stage apoptosis, as has been previously described for the squid–vibrio system (Foster and McFall-Ngai, 1998; Troll et al., 2009). To quantify early-stage apoptosis, animals were incubated in acridine orange for 5 min, washed for 1 min, and then anesthetized in 2% ethanol in FSIO. Each animal was then dissected on a depression slide and examined by confocal microscopy. The number of early-stage apoptotic nuclei in epithelium of the light organ appendages was counted. Nearby gut tissue was examined for non-specific apoptotic effects of the chemicals. To quantify late-stage apoptosis, animals were fixed as described above. They were then washed and the light organs were dissected out into mPBS. The light organs were permeabilized overnight and then subjected to rhodamine phalloidin staining as described above. TUNEL staining was done using the DEAEEnd Fluorometric Assay kit (Promega, Madison, WI, USA) using the previously described protocol (Troll et al., 2009). Light organs were mounted in VectaShield and visualized by confocal microscopy.

To score animals for regression, animals were incubated for 24 h in the presence or absence of inhibitor and the presence or absence of V. fischeri. At 24 h, the animals were moved into fresh vials without inhibitor or bacteria and then fixed at 48 h post exposure. Animals were fixed in 4% paraformaldehyde in FSIO overnight, washed 3 × 15 min in mPBS, and dehydrated in a series from 50–100% ethanol. The samples were desiccated in hexamethyldisilazane. Dried samples were mounted on plastic Petri dishes with colloidal graphite and the ventral mantle and siphon were dissected away to reveal the light organ epithelium, which was scored blindly for regression according to the previously developed scoring system (0 = full epithelial field visible, 1 = loss of medial epithelial ridge, 2 = shortening of anterior appendage, 3 = loss of posterior appendage, further shortening of anterior appendage, 4 = complete loss of ciliated field) (Doiño and McFall-Ngai, 1995).

Statistical significance of differences in apoptosis was analyzed using a one-tailed Student’s T-test followed by a Bonferroni adjustment of P-value for multiple comparisons.

Acknowledgements

We are grateful to Monica Tincher for her efforts in testing NOS inhibitors and NO donors in this study. We thank S. Peyer, Y. Wang and E. Ruby for their helpful comments on the manuscript. This work was funded by NSF IOS 0817232 to M.M.N. and E.G. Ruby, NIH R01-AI50661 to M.M.N., NIH RR R01-12294 to E.G. Ruby, and by NIH NRSA AI55397 to M.A.A. through the Microbes in Health and Disease Training Program.

References


© 2010 Blackwell Publishing Ltd, Cellular Microbiology


