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Attenuation of Host NO Production by MAMPs Potentiates Development of the Host in the Squid-Vibrio Symbiosis

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Summary

The presence of bacterial pathogens typically upregulates the host's production of nitric oxide synthase (NOS) and nitric oxide (NO) as antimicrobial agents. This dramatic response 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 an attenuation of host NOS/NO, which occur in high levels in the hatchling light organ. In the present study, 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 visualized and quantified the levels of NOS with immunocytochemistry and NO with a NO-specfic fluorochrome. When added exogenously to seawater containing hatchling 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 the 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 (*i.e.*, beneficial or pathogenic bacteria). These data also provide a mechanism by which symbiont MAMPs regulate host development through NO attenuation.

Keywords

Nitric oxide; *Euprymna scolopes*; *Vibrio fischeri*; Photobacterium; Aliivibrio; symbiosis; apoptosis; lipopolysaccharide; peptidoglycan; tracheal cytotoxin; host-microbe interactions

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Introduction

Nitric oxide (NO) is involved in a wide array of biological processes in animals, including cell signaling, neurotransmission, and innate immunity. As an easily diffusible gas, NO acts as an intra- and intercellular signaling molecule (reviewed in Martinez-Ruiz *et al.*, 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; Ferguson and Mathesius, 2003; 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.*, 2010). The nocturnal Hawaiian bobtail squid, *Euprymna scolopes* (Fig. 1A), apparently uses the luminous Gram-negative bacterium, *Vibrio fischeri*, as part of an anti-predatory strategy (Jones and Nishiguchi, 2004; reviewed in Stabb and Millikan, 2009). The bacteria are horizontally transmitted and so the juvenile squid must harvest the bacteria from the surrounding seawater anew each generation. 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 remodeling of the surface epithelium of the light organ and the attenuation of nitric oxide (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 LPS, begins around 6–12 hours post-infection. Late-stage apoptosis, which is characterized by DNA fragmentation, begins at 12–14 hours post exposure and continues until the epithelium is completely regressed. This later process is induced synergistically by the peptidoglycan (PGN) monomer 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 peptidoglycan-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 animals 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 MAMPs-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. S1). 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 labeled with the uNOS antibody, which were used as internal controls (Fig. S2). Control APO animals had statistically significantly higher levels of uNOS antibody labeling in the cells lining their ducts and antechambers as compared to 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-amino-5-methylamino-2′,7′-difluorofluorescein) an indicator of NO. The changes in NO levels under the different conditions correlated with changes in uNOS antibody labeling. High levels of NO production in the light organ characteristic of APO animals were attenuated by the onset of colonization with *V. fischeri* (SYM). We found that 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$^-$ bacteria had NOS labeling 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 were unable to induce NOS attenuation (ltg$^-$ + empty vector) 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 gonorrheae*, *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. 4D). 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

LPS 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 NO inhibitor, S-methyl-thiocitrulline (SMTC), and measured its effects on both early- and late-stage apoptosis. We found that, at effective working concentrations (Fig. S3A, B), SMTC induced an increase in the labeling 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,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 regression of the ciliated epithelium in SYM animals that were exposed to inhibitor as compared to unexposed SYM controls (Fig. 5C). We also found that late-stage apoptosis increased with the addition of either LPS or TCT and the NO inhibitor as compared to LPS or TCT untreated controls. LPS and TCT typically do not induce late-stage apoptotsis 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 analyzed 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-acetylpenacillamine (SNAP). We found that SNAP, at effective working concentrations (Fig. S3A,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. 5 A,B). We confirmed by confocal microscopy the presence of increased NO in target tissues of treated animals (Fig. S3A). 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 bacteria-induced developmental processes increase the scope of biologists' view 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: 1) NOS and NO production are regulated by the synergistic activity of the symbiont MAMPs, LPS and TCT; 2) mutants deficient in TCT release are defective in normal NOS reduction; 3) NOS reduction is not specific to *V. fischeri* LPS in combination with TCT; and 4) NO attenuation is an intermediary in the induction of apoptosis of symbiosis-associated tissues, which was previously shown to be induced by symbiont MAMPs in this system, although the data suggest that NO attenuation is not the sole inducer.

The bacteria-induced decrease in NOS/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 (Hauschildt *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 has been shown to act 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 NOS/NO production. Most regulation of NOS 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, NOS 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 NOS and NO levels, it is possible that NOS is regulated transcriptionally. However, several NOS-regulating proteins, NOSTRIN and NOSIP, have been found in the light organ EST database (Chun *et al.*, 2006), and NOS/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 peptidoglycan 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 NOS, apoptosis, and morphogenesis (Goodson *et al.*, 2005, Troll *et al.*, 2009).

NOS 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 *V. cholera*, *E. 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.*, 2010, Wang, presonal communication)

Pharmacological inhibition of NO induced early-, but not late-stage apoptosis, while artificially supplementing 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 *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-vibro 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 NO 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.

Studies with MAMPs, alone and in combination, as well as with NOS inhibitors and NO donors, have provided insight into the underlying mechanisms of bacteria-induced host tissue development. The data resulting from this study provide evidence that: 1) NOS attenuation is necessary but insufficient to trigger late-stage apoptosis; and 2) LPS and TCT are able to induce late-stage apoptosis individually in the presence of the NO inhibitor, where under natural conditions, 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 NOS/NO turndown, which would induce the host epithelia 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). Future studies of the system will be aimed at unraveling the complex interplay between these various elements.

Methods

General Methods

Adult *E. scolopes* were caught off the coast of Oahu and bred in salt water tanks as previously described (Mongomery and McFall-Ngai, 1993). Juveniles obtained from this breeding colony were collected within 15 min of hatching and washed three times in filter sterilized instant ocean (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 *E. scolopes* with (SYM) and without (APO) the symbiotic *V. fischeri*, as well as juveniles colonized with specific *V. fischeri* mutants. APO juveniles were maintained in FSIO free of *V. fischeri* over the course of the experiment. SYM animals and those exposed to mutant strains of *V. fischeri* were maintained in FSIO with 5,000 CFU/ml of wild-type (WT) *V. fischeri* strain ES114 or the indicated mutant strain overnight. Strains of *V. fischeri* that are defective in TCT release consisted of a mutant ltg$^-$ (ES114-derivative strain DMA388: Δ*ltgA*, Δ*ltgD, ltgY::erm*) and its complemented strains (ltg$^-$+*ltgA*, ltg$^-$+*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 μM 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 of LPS derived from *V. fischeri* or other species and/or 1 μM 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. S1). 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) 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 μM 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) 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, 0.45M sodium chloride, pH 7.4) at 4°C. Light organs were removed from the squid and permeabolized for 48 h in 1% Triton-X-100 in mPBS. Then, the light organs were blocked overnight at 4°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 d at 4°C. Samples were rinsed 4 × 1 h in 1% Triton-X-100 in mPBS and incubated overnight in blocking solution at 4°C. Samples were then incubated with a 1:50 dilution of fluorescein-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoReasearch Labs, West Grove, PA) 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 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 photobleaching 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-penacillamine (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 effect the animals' behavior or survival, but still elicited a level of NO, measured by DAF-FM staining, in SYM animals that was comparable to APO animals unexposed controls (Fig. S3A,C). Squid exposed to the NO inhibitor S-methyl-thiocitrulline, which is stable in solution, were exposed to a concentration of 100 μM for 18 h. To determine the amount of SMTC to use, we exposed the squid to several concentrations and found the concentration that did not appreciably effect the animals' behavior or survival, but still elicited a level of NO, measured by DAF-FM staining, in APO that was comparable to SYM unexposed controls (Fig. S3A–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 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 were 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 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 DEADEnd Fluorometric Assay kit (Promega, Madison, WI) 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 hours in the presence or absence of inhibitor and the presence or absence of *V. fischeri*. At 24 hours, the animals were moved into fresh vials without inhibitor or bacteria and then fixed at 48 hours 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) (Doino *et al.*, 1995).

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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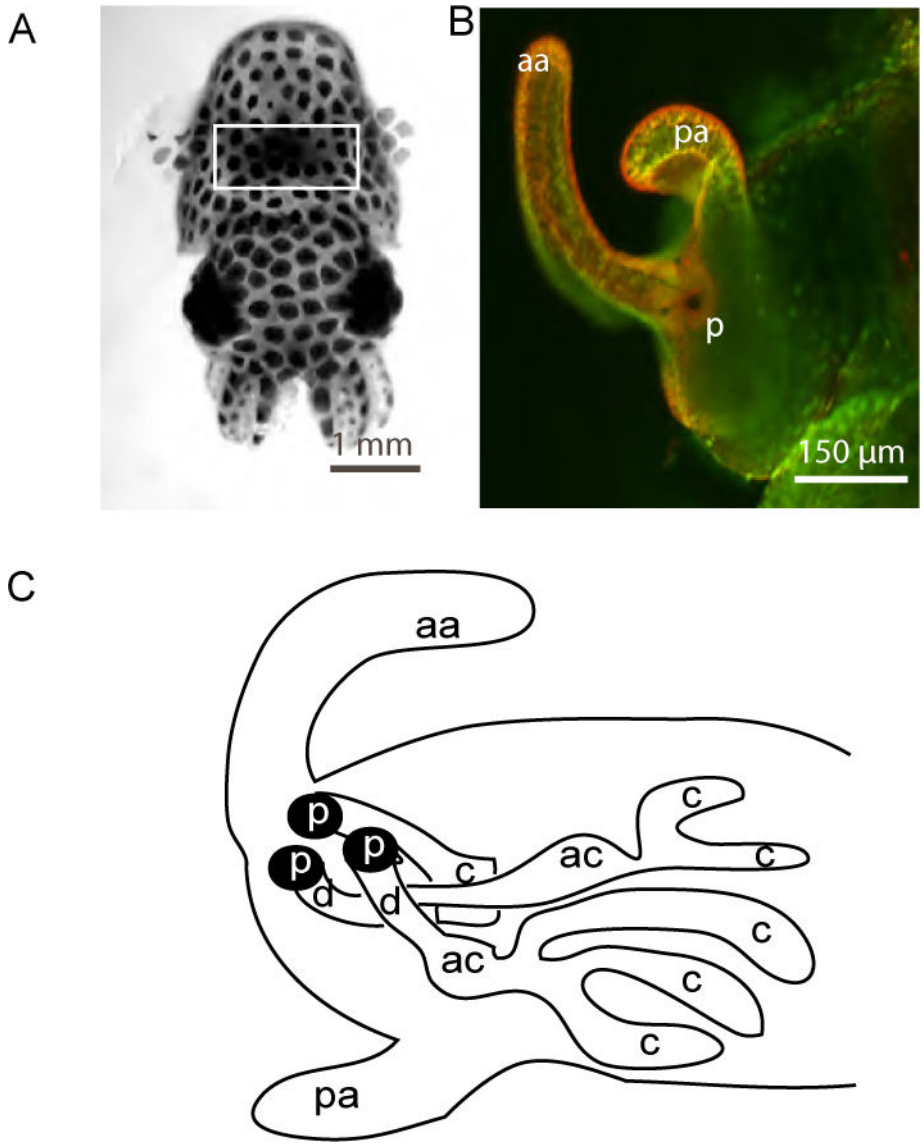

Fig 1. Juvenile *E. scolopes* and its light organ

A. Left: A dorsal view of a juvenile *E. scolopes* showing the location of the light organ beneath the mantle (box). B. An LSM confocal micrograph of the surface of a hatchling light organ stained with acridine orange, which reveals the general morphology of the organ. Each lateral surface (box) is covered by a complex, juvenile specific superficial ciliated epithelium C. An illustration of the internal morphology of the light organ. aa = anterior appendage, ac = antechamber, c = crypt, d = duct, p = pores, pa = posterior appendage

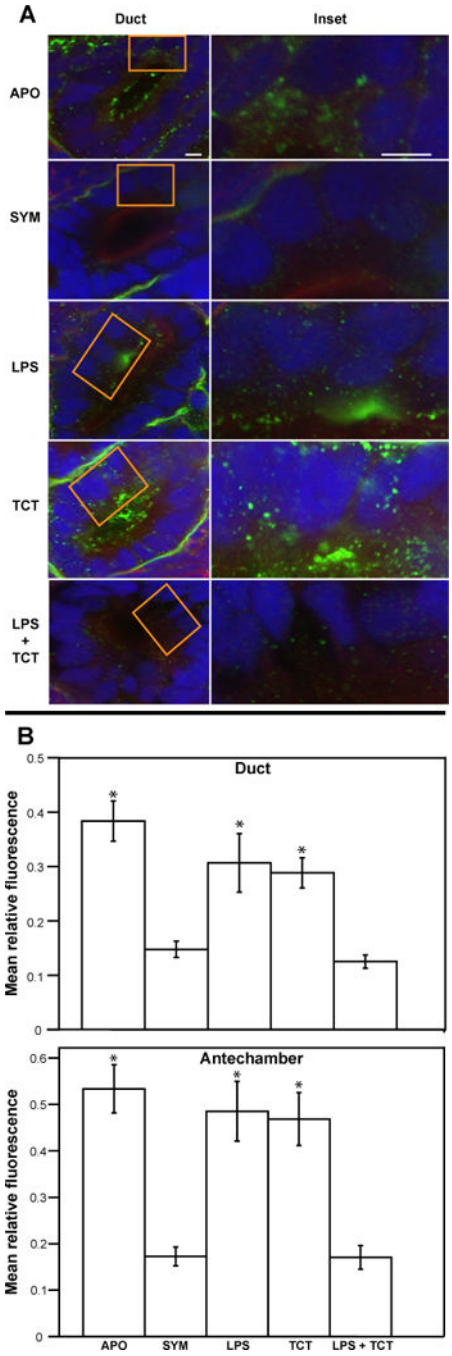

Fig 2. The visualization and quantification of NOS attenuation in the presence and absence of *V. fischeri* MAMPs by LSM confocal microscopy

A. Representative micrographs of duct cells labeled 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 Materials and Methods) of a single representative microscopy experiment (APO n=10, SYM

n=12, LPS n=8, TCT n=9, LPS+TCT n=14). Bars, standard error. (*), data points that were significantly different from SYM.

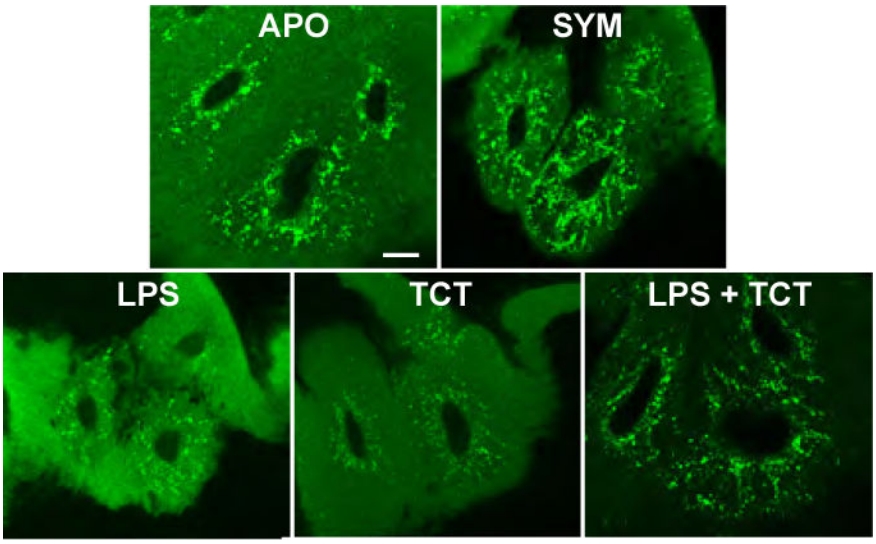

Fig 3. The visualization by LSM confocal microscopy of NO attenuation in the presence and absence of *V. fischeri* MAMPs

Lower images, changes in NO levels due to bacterial products. Upper images, control aposymbiotic and symbiotic tissues. Representative micrographs of duct cells stained for NO production with DAF-FM (green). Bar, 20 μm.

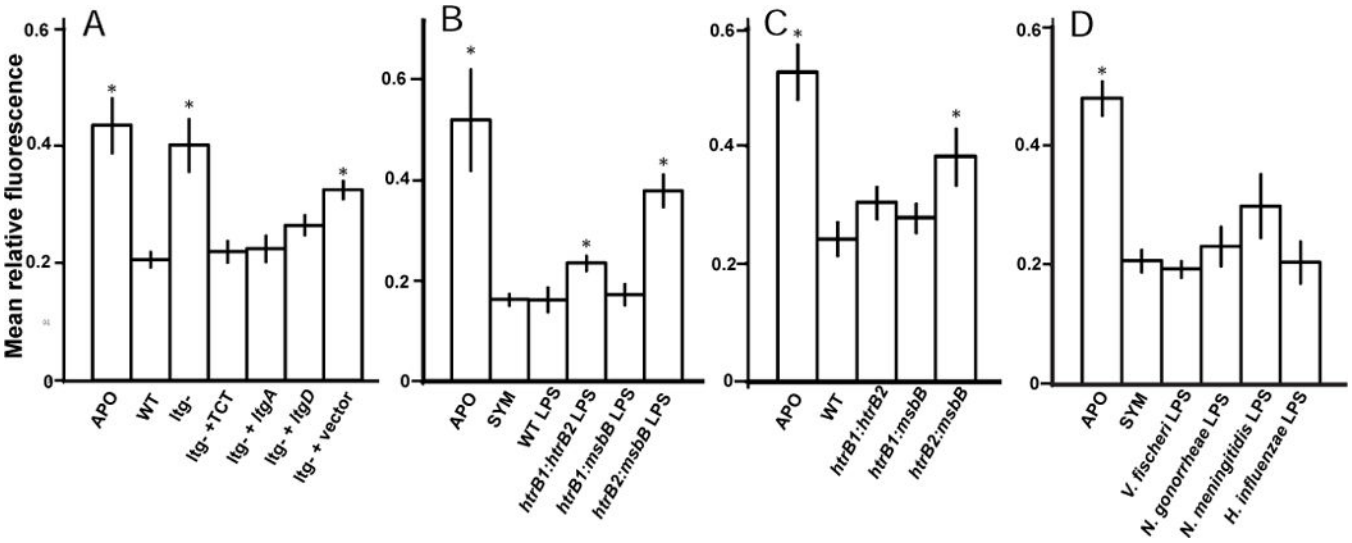

Fig 4. The visualization by LSM confocal microscopy and quantification of NOS attenuation in the presence of *V. fischeri* MAMP mutants and in the presence of LPS isolated from non-symbiotic bacteria

A. Quantification of antechamber cells stained with a commercial uNOS antibody. Animals were exposed to mutants defective in TCT release (ltg$^-$) or complemented pharmacologically with 1 μM TCT (ltg$^-$ + TCT) or genetically with one of the lytic transglycosylase genes in a multicopy plasmid (ltg$^-$ + *ltgA* or ltg$^-$ + *ltgD*). (APO n=9, SYM n=13, ltg$^-$n=8, ltg$^-$+TCT n=12, ltg$^-$+*ltgA* n=14, ltg$^-$+*ltgD* n=11, ltg$^-$+plasmid n=14) B. Quantification of antechamber cells stained with a commercial antibody from animals exposed to LPS purified from non-symbiotic bacteria (*N. gonorrheae*, *N. meningitides*, *H. influenzae*) and 1 μM TCT (APO n=6, SYM n=7, *V. fischeri* LPS n=9, *N. gonorrheae* n=9, *N. meningitides* n=6, *H. influenzae* n=8). Graphs are of single representative experiments. [Bars, standard error. (*), data points which were significantly different from SYM.]

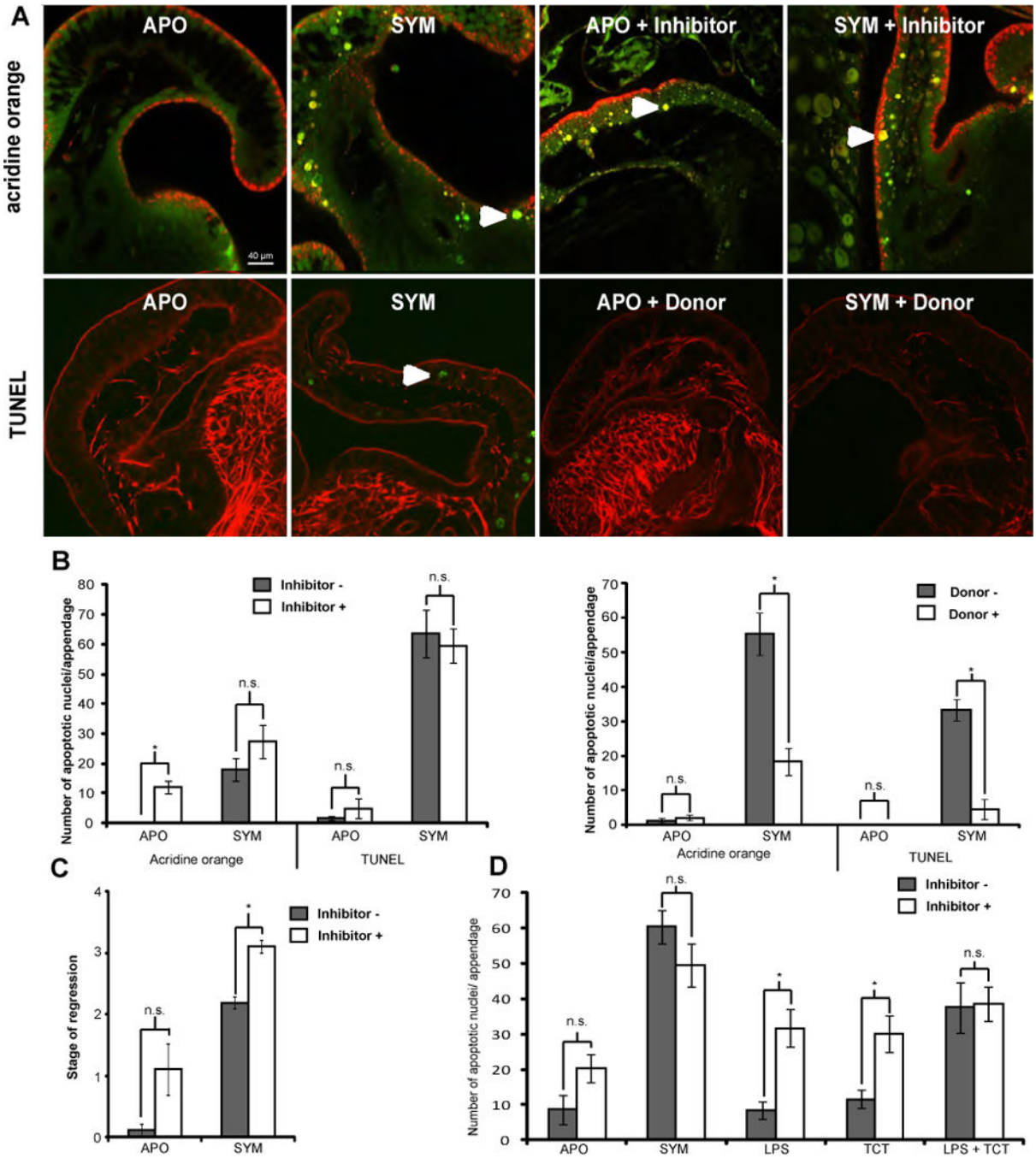

Fig 5. The role of NO in the induction of apoptosis

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 were counted per appendage for each treatment. C. Graph of single representative experiment of animals exposed to NO inhibitor (SMTC) and scored for stage

of regression. D. Graphs of average of two experiments of animals exposed to NO inhibitor (SMTC) and/or bacterial MAMPs as indicated and quantified for late-stage (TUNEL) apoptosis. The number of apoptotic nuclei were 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. (*), significance of $p \leq 0.05$. n.s., "not siginificantly different".

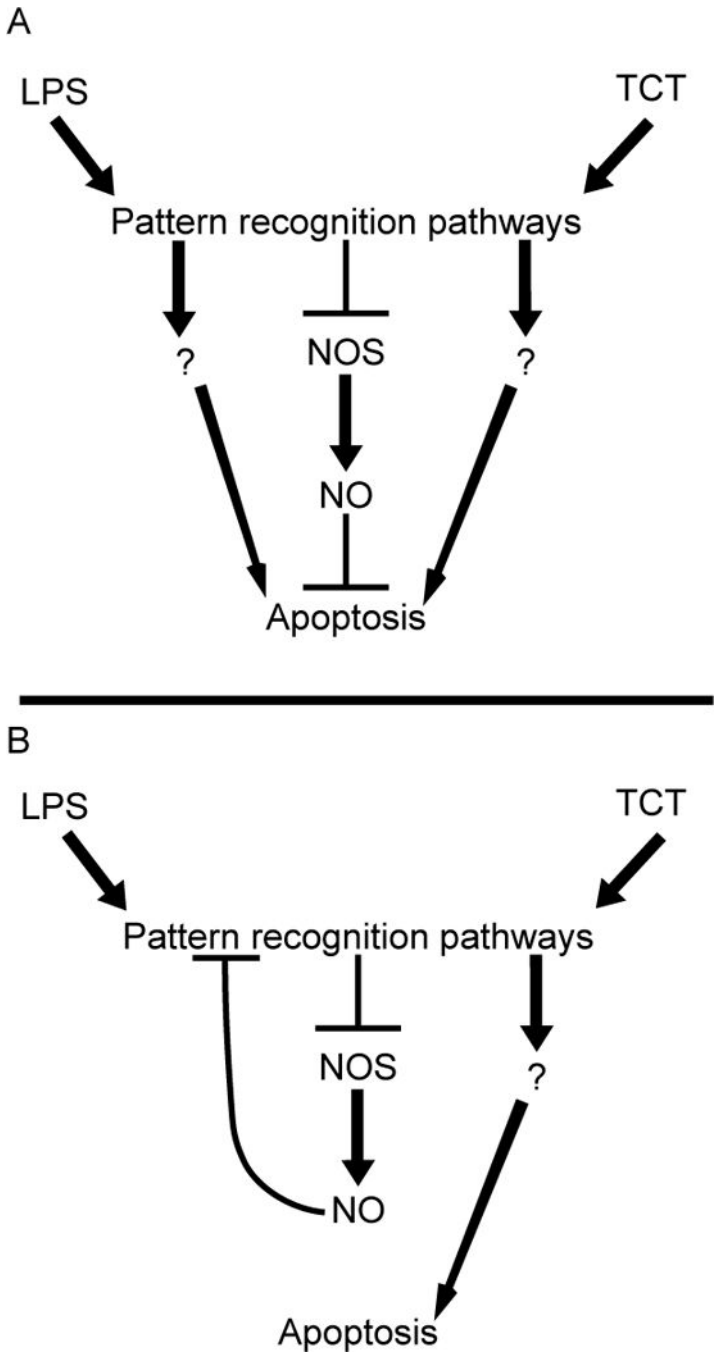

Fig 6. A model for the role of NOS/NO in developmental apoptosis of the light organ

In stage 1, LPS and TCT are recognized by the animal and have the synergistic effect of attenuating NOS/NO, which removes a block on early-stage apoptosis. Removal of this block allows late-stage apoptosis (stage 2) to proceed by LPS or TCT stimulating an as yet undetermined pathway, possibly p53.