

Nitric oxide and cnidarian bleaching: an eviction notice mediates breakdown of a symbiosis

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Summary

Nitric oxide (NO) is a free radical implicated in numerous cell signaling, physiological and pathophysiological processes of eukaryotic cells. Here, we describe the production of NO as part of the cellular stress response of the symbiotic sea anemone *Aiptasia pallida*, which hosts dinoflagellates from the genus *Symbiodinium*. We show that exposure to elevated temperatures induces symbiotic anemones to produce high levels of NO, leading to the collapse of the symbiosis. These results shed light on the poorly understood cellular mechanism through which elevated seawater temperature causes the release of symbiotic algae from symbiotic cnidarians, a detrimental process known as coral (cnidarian) bleaching. The results

presented here show that the host cell is a major source of NO during exposure to elevated temperatures and that this constitutes a cytotoxic response leading to bleaching. These results have important evolutionary implications as the observed NO production in these basal metazoans displays many parallels to the cytotoxic inflammatory response to pathogens, a well-understood process in mammalian model systems.

Key words: *Aiptasia pallida*, nitric oxide, peroxyxynitrite, oxidative stress, *Symbiodinium*, zooxanthellae, coral bleaching, Cnidaria, dinoflagellate, innate immunity, programmed cell death, necrosis, apoptosis, symbiosis.

Introduction

Many cnidarians symbiotic with the intracellular dinoflagellate *Symbiodinium* sp., such as the ecologically important reef-building corals, lose their algal partners in response to a variety of stresses, including exposure to elevated temperature and excessive visible and UV radiation (Gates et al., 1992; Glynn and D'Croz, 1990). This process, known as coral (cnidarian) bleaching, is often lethal to the host and can have devastating ecological effects on the coral reef ecosystem (Glynn, 1996; Hoegh-Guldberg, 1999). In cnidarian–dinoflagellate symbioses, exposure to elevated temperature can result in inhibition of algal photosynthesis and increased production of reactive oxygen species (ROS) such as superoxide radical (Franklin, 2004; Lesser, 1996; Lesser et al., 1990; Perez et al., 2001). The increase in ROS production is hypothesized to increase cellular damage and lead to bleaching; however, the cellular and molecular mechanisms leading to the loss of algae is poorly understood.

Cnidarian bleaching is known to be mediated in part through apoptosis and necrosis leading to the loss of integrity of gastrodermal tissue that contains the algae (Dunn et al., 2004). However, the events that lead to host cell death have not been studied in detail. In other systems, an important mediator of cell death due to oxidative stress is the reactive

nitrogen species peroxyxynitrite, which is a reaction product of superoxide and nitric oxide (NO) (Radi et al., 2000). In animals, NO is produced by a family of enzymes known as nitric oxide synthases (NOS; EC 1.14.13.39). These enzymes catalyze the conversion of arginine, NADPH and O₂ to NO, citrulline and NADP⁺. Early evidence suggests that NO is produced in symbiotic cnidarians and that it may play a role in bleaching. In the symbiotic anemone *Aiptasia pallida*, an arginine-dependent NOS-like activity has been reported (Trapido-Rosenthal et al., 2001). Further, NO was produced in freshly isolated algae from the coral *Madracis mirabilis* and was implicated in the bleaching process (Trapido-Rosenthal et al., 2005).

We were interested, therefore, in further investigation of NO in symbiotic cnidarians and its potential cellular role in the bleaching process. We visualized production of NO *in vivo* as a function of heat stress in the symbiotic anemone *Aiptasia pallida*, using laser scanning confocal microscopy and the NO-sensitive fluorescent dye DAF-FM (Nagano and Yoshimura, 2002). We present evidence in support of a model in which the host cell increases its production of NO as a function of an increased oxidative load during exposure to elevated temperatures. We suggest that this constitutes a cytotoxic response that leads to bleaching.

Materials and methods

Maintenance of Aiptasia pallida and Symbiodinium cultures

Cultures of *Aiptasia pallida* (Verrill) from the Florida Keys, FL, USA were maintained in artificial seawater (Instant Ocean®; Aquarium Systems, Mentor, OH, USA) at 25°C and 12 h:12 h light:dark photoperiod with a light irradiance of approximately 70 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Aposymbiotic anemones were obtained by the cold stress technique (Muscatine et al., 1991) and kept in the dark. Algae from the Florida anemones (*Symbiodinium* sp.) were isolated by homogenizing anemones in a glass tissue grinder followed by repeated centrifugations and subsequent resuspension in sterile seawater. The algae were then placed in 50 ml tubes with sterile Guillard's f/2 enriched seawater culture medium (Sigma, St Louis, MO, USA). Algal cultures were kept in the same light and temperature conditions as the symbiotic anemones.

Confocal microscopy

For preparation for confocal microscopy, the media of experimental anemones was replaced with relaxing solution of a 1:1 ratio of 37 mmol l^{-1} MgCl_2 :seawater (35%) with 3 $\mu\text{l ml}^{-1}$ of the NO probe DAF-FM-DA (Molecular Probes, Eugene, OR, USA) (Nagano and Yoshimura, 2002). After incubating the anemones for at least 30 min in the dark, the media was removed and a few drops of melted 1% low-melting agarose in relaxing solution cooled to 30°C was added to immobilize the anemones. After allowing the agarose to gel for 1 min, 3 ml of relaxing media was added on top of the agarose-embedded anemones. Unstained control anemones were processed as above without adding the probe to the mix. These were used to control for possible host tissue autofluorescence. Cultured algae were processed by incubating 1 ml of suspension with 1 $\mu\text{l ml}^{-1}$ DAF-FM-DA for at least 30 min before observing on a glass slide and cover slip. The samples were observed under a Zeiss LSM510 Meta microscope (The Center for Genome Research and Biocomputing at Oregon State University) with a 40 \times /0.8 3.2 mm working distance water objective lens. Excitation was provided by an argon laser at 488 nm to excite the DAF-FM probe and a HeNe543 laser to excite chlorophyll autofluorescence. DAF-FM NO-dependent fluorescence was detected using a 510–530 nm filter and chlorophyll autofluorescence using the metadetector at 600–700 nm. Each excitation wavelength (488 and 543 nm) was used separately on different scans using the multiscan function. Before image scanning, the focal plane of the optical section was adjusted to include the gastrodermal layer of tentacles. All images were obtained with the same software scanning settings including detector gain and laser intensity settings. After visualization, samples that showed no NO-dependent fluorescence were subsequently incubated for 30 min in the NO donor sodium nitroprusside (1 mmol l^{-1} SNP; Sigma, St Louis, MO, USA), and fluorescence was measured to confirm successful loading of the dye. Quantification of NO-dependent DAF-FM fluorescence was

achieved by first defining the gastrodermal portions as regions of interest and measuring the average pixel intensity value for that region with the LSM 5 software.

Experiments

To test the effect of heat stress on NO production, 3–5 small aposymbiotic and symbiotic anemones (5–10 mm long) were each placed in 3 ml of artificial seawater in 5 ml Petri dishes. The Petri dishes were modified by fitting them with a glass cover slip adhered to a hole drilled on the bottom. The anemones were pre-incubated overnight at 25°C. After the pre-incubation, the anemones were incubated in either 25°C or 33°C for 24 h on a 12 h:12 h light:dark photoperiod. Algal cultures (50 ml) were similarly heat stressed. After the incubation, the anemones and algal cultures were processed for confocal microscopy. To examine the effect of an NOS inhibitor on NO production after heat stress, heat-stressed or control symbiotic anemones were incubated for 10 min in 10 mmol l^{-1} L-aminoguanidine (LAG; Sigma) before processing for confocal microscopy. Aposymbiotic anemones that served as a positive control for host NOS activity were incubated for 5 h in 1 $\mu\text{g ml}^{-1}$ lipopolysaccharide (LPS; Sigma) before preparation for confocal microscopy. Some LPS-treated anemones were also pre-incubated in LAG as above.

To test the effect of 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU; Sigma) on NO production, anemones or cultured algae were incubated in 50 $\mu\text{mol l}^{-1}$ DCMU for 24 h. Samples were then prepared for confocal microscopy.

Bleaching experiments were carried out by incubating medium-sized (0.5–1 cm oral disc diameter) symbiotic anemones at 25°C with 1.0 mmol l^{-1} SNP with or without 1 mmol l^{-1} of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma) or at 33°C with or without 20 mmol l^{-1} cPTIO for 24 h. Controls were included and each anemone was incubated individually in 5 ml Petri dishes. Bleaching was quantified using methods similar to those previously published (Perez et al., 2001). Briefly, both algae expelled into the media from anemones and those remaining in the host tissues (*in hospite* algae) were counted. Percent bleaching was calculated as the number of expelled algae/(expelled algae + number of *in hospite* algae). In a separate experiment, small anemones were either heat-stressed with or without 20 mmol l^{-1} cPTIO at 33°C for 24 h or incubated with 1.0 mmol l^{-1} SNP with or without 20 mmol l^{-1} cPTIO for 3 h. Treated anemones as well as control anemones (also with or without 20 mmol l^{-1} cPTIO) were then rinsed with seawater and prepared for confocal microscopy using DAF-FM to measure NO-dependent fluorescence as described above.

Results

Heat stress triggers production of nitric oxide in symbiotic A. pallida

To determine if NO was differentially produced as a function of heat stress in *A. pallida*, we incubated symbiotic

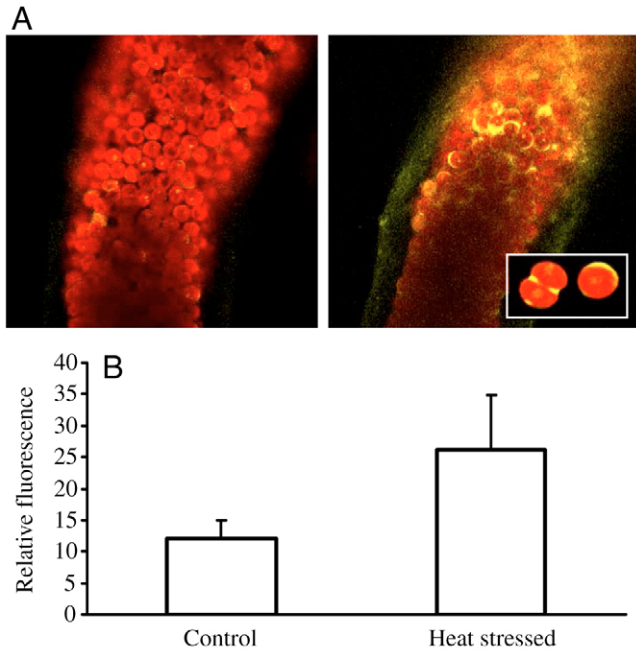


Fig. 1. Heat stress induces fluorescence of the NO-sensitive probe DAF-FM in *A. pallida*. (A) Optical cross-section through a tentacle of symbiotic *A. pallida* incubated at an ambient temperature of 25°C (left) and after 24 h of heat stress at 33°C (right), with inset showing expelled host cells with algae. The DAF-FM (510–530 nm) NO-dependent fluorescent signal is colored yellow; algal autofluorescence is colored red. (B) Quantification of relative fluorescence intensity of NO-sensitive DAF-FM (510–530 nm) in tentacles as a function of heat stress ($N=3$ anemones per treatment; bars represent means + s.d.; t -test, $P=0.0015$).

anemones at 33°C for 24 h and observed their tentacles using laser scanning confocal microscopy after loading the animal with the NO-sensitive fluorescent probe DAF-FM-DA (Nagano and Yoshimura, 2002). Exposure to elevated temperature resulted in a significant twofold increase in NO-dependent DAF-FM fluorescence in tentacles of symbiotic anemones (Fig. 1; t -test, $P=0.0015$). The fluorescent NO-sensitive signal was found largely in the host gastrodermal cells containing the algae, both in the intact tissue as well as in the host cells released during bleaching (Fig. 1A inset). The difference in the fluorescent signal between control and heat-stressed anemones was not due to unequal loading of the dye as a function of temperature treatment since addition of exogenous NO to controls, by incubation with the NO donor sodium nitroprusside (SNP), always resulted in an increase of the NO-dependent fluorescent signal (data not shown). Unstained controls did not show autofluorescence of host tissues.

The host *A. pallida* as a source of NO during heat stress

To examine more closely the source of NO during exposure to elevated temperature, we performed the heat stress experiments on aposymbiotic anemones and cultured *Symbiodinium* from *A. pallida* loaded with DAF-FM. We did

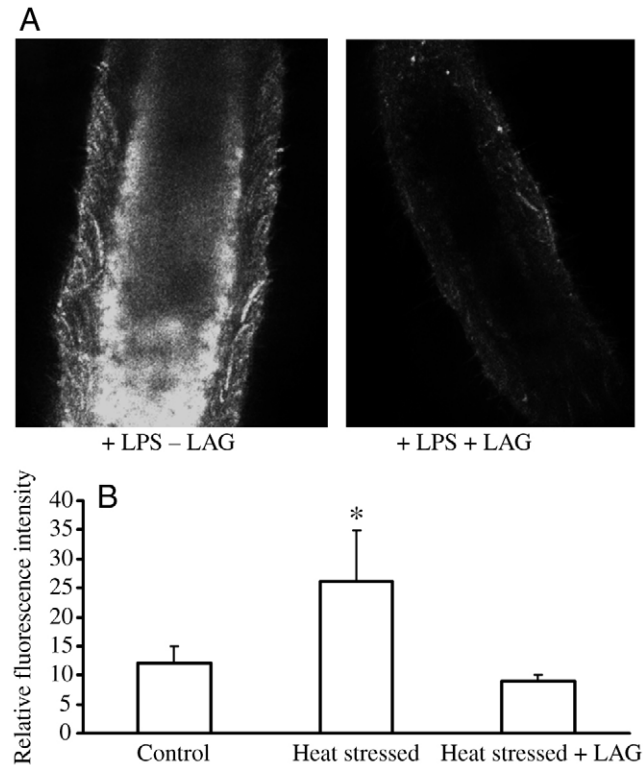


Fig. 2. The NOS inhibitor L-aminoguanidine (LAG) inhibits both lipopolysaccharide (LPS) and heat stress-induced NO production in *A. pallida*. (A) Gray-scale rendering of confocal images of DAF-FM-loaded tentacles of aposymbiotic anemones incubated for 5 h in 1 $\mu\text{g ml}^{-1}$ LPS alone (left) or in LPS with 10 mmol l^{-1} LAG (right). (B) Quantification of DAF-FM fluorescence as a function of heat stress alone and in the presence of 10 mmol l^{-1} LAG ($N=3$ anemones per treatment; bars represent means + s.d.). Heat-stressed anemones showed a significantly greater (*) DAF-FM fluorescence than control or heat-stressed anemones incubated in LAG (ANOVA, $P=0.006$; Tukey HSD, $P=0.021$).

not observe NO production in either aposymbiotic animals or cultured algae in isolation as a function of heat stress. This suggests that the partners in combination are required to cause the production of NO.

Production of NO through NOS-like activity was tested by using the NOS inhibitor LAG (Danielisova et al., 2004). To induce NOS-like activity in anemones, we incubated aposymbiotic anemones in LPS (1 $\mu\text{g ml}^{-1}$), which is known to cause the upregulation of inducible NOS in vertebrate systems (Ganster and Geller, 2000). LPS induced NO production in gastrodermal tissues of aposymbiotic *A. pallida* at similar levels to those observed in heat-stressed symbiotic anemones (Fig. 2A). Incubating LPS-treated anemones with LAG (10 mmol l^{-1}) resulted in strong inhibition of NO production (Fig. 2A). To test whether the heat-stress-induced NO production observed in symbiotic anemones was due to a host NOS inhibitable by LAG, we incubated heat-stressed anemones in 10 mmol l^{-1} LAG and measured NO production. Incubation of heat-stressed symbiotic anemones in the NOS

inhibitor also significantly inhibited NO production due to heat stress (Fig. 2B; ANOVA, $P=0.006$; Tukey HSD, $P=0.021$). This suggests that in response to exposure to elevated temperature, symbiotic anemones produce NO through a host-regulated NOS-like activity.

Oxidative stress can trigger the production of NO in symbiotic anemones

In vertebrate systems, oxidative stress is another well-known signal for the upregulation of inducible NOS (Mendes et al., 2003). We hypothesized that production of ROS by the algae, a phenomenon known to occur during heat stress, would result in NO production. To test for NO production due to elevated oxidative stress, we incubated symbiotic and aposymbiotic anemones as well as cultured algae in DCMU, an inhibitor of photosynthetic electron transport known to increase production of ROS and trigger bleaching (Franklin et al., 2004; Lesser, 1996). Incubating symbiotic anemones with $50 \mu\text{mol l}^{-1}$ DCMU elicited a nearly threefold increase in NO production in tentacles similar to that seen in heat-stressed anemones while DCMU did not have a significant effect in aposymbiotic anemones (Fig. 3; t -test, $P=0.0041$). Further, we could not detect production of NO in cultured algae in the presence of DCMU (data not shown). These results suggest that anemones produce NO in response to conditions known to elicit oxidative stress in the algae.

Nitric oxide induces cnidarian bleaching in *A. pallida*

To test whether NO plays a role in bleaching, we incubated anemones with the NO donor SNP at room temperature and measured the release of algae from host tissues. SNP-derived NO caused a significant increase in bleaching, increasing from less than 1% in controls to 17% at 1 mmol l^{-1} SNP (Fig. 4A; t -test, $P=0.012$). Further, the effect of SNP on bleaching was significantly decreased by co-incubation with the NO scavenger cPTIO (Fig. 4A; t -test, $P=0.0368$). In addition, incubating anemones in 20 mmol l^{-1} cPTIO during exposure to

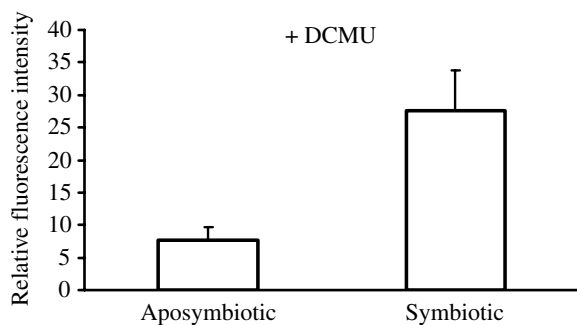


Fig. 3. DCMU increases NO production in symbiotic *A. pallida*. Quantification of DAF-FM fluorescence of tentacles from aposymbiotic and symbiotic anemones after a 24 h incubation in $50 \mu\text{mol l}^{-1}$ DCMU ($N=3$ anemones per treatment; bars represent means + s.d.; t -test, $P=0.0041$).

elevated temperature (33°C) significantly decreased bleaching by 50% (Fig. 4B; t -test $P=0.0176$). The NO-dependent DAF-FM fluorescence significantly increased in anemones incubated with 1 mmol l^{-1} SNP as well as anemones exposed to elevated temperature (Fig. 4C; two-way ANOVA, $P<0.001$; Tukey HSD, $P<0.05$). Co-incubation of both SNP and heat-stressed anemones with 20 mmol l^{-1} cPTIO effectively decreased NO-dependent DAF-FM fluorescence to levels found in control anemones (Fig. 4C; two-way ANOVA, $P<0.001$; Tukey HSD, $P<0.05$).

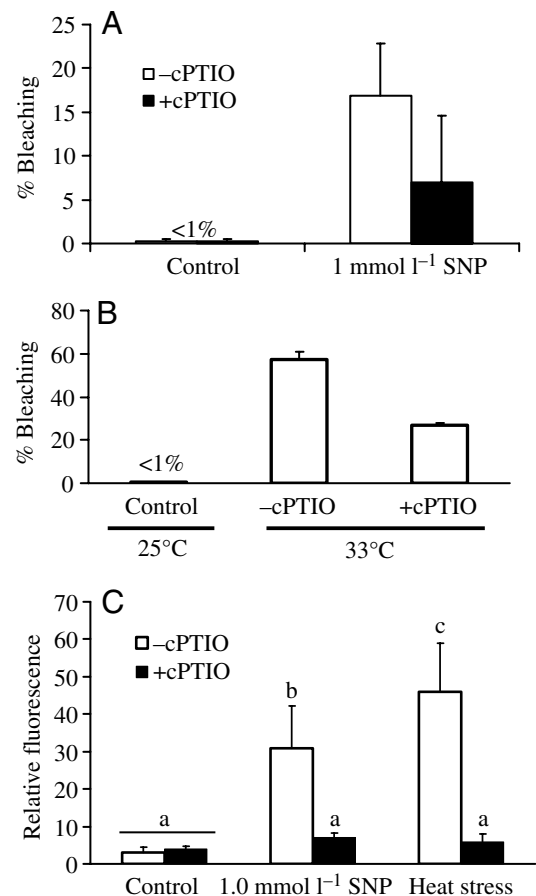


Fig. 4. Nitric oxide mediates bleaching in *A. pallida*. (A) Bleaching (% expulsion; bars represent means + s.d.; $N=3$ anemones per treatment) of anemones incubated for 24 h at 25°C with the NO donor SNP (1 mmol l^{-1}) with (filled bars) or without (open bars) the NO scavenger cPTIO (1 mmol l^{-1}). Control anemones (without SNP) released <1% of their algae. Incubation with SNP resulted in a significant increase in bleaching (t -test, $P=0.021$) while co-incubation with cPTIO significantly decreased this effect (t -test, $P=0.0368$). (B) Bleaching (% expulsion; bars represent means + s.d.; $N=3$ anemones per treatment) of anemones incubated for 24 h at 25°C (control) or 33°C , with or without cPTIO (20 mmol l^{-1}). There was a significant difference between the two heat-stress treatments (t -test, $P=0.0176$). (C) NO-dependent DAF-FM fluorescence in control, heat-stressed or SNP-treated anemones with or without 20 mmol l^{-1} cPTIO. Bars sharing the same letter are not significantly different (two-way ANOVA, $P<0.001$; Tukey HSD, $P<0.05$).

Discussion

This study presents two major findings. Host cells of symbiotic *A. pallida* produce NO as a function of heat stress, possibly in response to the resulting increase in oxidative load. Secondly, NO produced during exposure to elevated temperature mediates bleaching.

Symbiotic anemones exposed to elevated temperatures produced NO while aposymbiotic anemones do not. There are two possible (but not mutually exclusive) explanations for this result. Either symbiotic algae in the host are responsible for the production of NO or the host produces NO in response to heat-stressed algae. There are several lines of evidence that support the second scenario. First, we did not detect any production of NO in cultured algae exposed to elevated temperatures. The algae, however, could be producing NO during heat stress only during symbiosis, perhaps in response to an unknown host-derived factor. But, to date, no homologous NOS-encoding genes or cDNAs have been discovered in plants or lower eukaryotes including apicomplexans, a sister taxon to dinoflagellates (Chandok et al., 2003; Gardner et al., 2002; Guo et al., 2003). It is possible that NO could be produced by NOS-independent mechanisms such as by the enzyme nitrate reductase, as has been shown in the chlorophyte *Chlamydomonas* (Sakihama et al., 2002). If the symbionts are producing NO through an NOS-independent activity, then using a classic NOS inhibitor such as LAG should not inhibit NO production of heat-stressed symbiotic anemones. However, we were able to drastically inhibit NO production with LAG at concentrations that also inhibited LPS-induced NO production in aposymbiotic anemones (Fig. 2). In other systems, LPS has been demonstrated to induce the upregulation of the inducible form of NOS (iNOS) transcription through nuclear factor κ B (NF κ B)-dependent signaling as part of the inflammatory response of mammalian phagocytes in response to bacterial pathogens (Ganster and Geller, 2000). This pathway has not been described in cnidarians. In a recent publication, preparations of expelled as well as freshly isolated algae from the coral *Madracis mirabilis* showed increased NOS activity during heat stress, as measured by the conversion of arginine to citrulline (Trapido-Rosenthal et al., 2005). This apparently algal NOS-like activity may be explained by host cell contamination that, in our experience, is unavoidable in such preparations. Expelled material during bleaching also typically contains numerous gastrodermal cells containing algae (Gates et al., 1992), which we have observed to produce NO as well (Fig. 1A inset). Taken together, this evidence suggests that the host is responding to heat-stressed algae by producing NO.

Heat stress inhibits photosynthesis while stimulating ROS production in the algae (Lesser, 1996). Inducing oxidative stress in the algae by blocking the electron transfer from photosystem II with the use of DCMU (Franklin et al., 2004; Lesser, 1996) also led to the production of NO in symbiotic anemones only but not in cultured algae or aposymbiotic anemones. Like LPS, oxidative stress is known to lead to upregulation of iNOS through the activation of the

transcription factor NF κ B (Mendes et al., 2003). Interestingly, *Plasmodium berghei*, an apicomplexan parasite (a sister taxon of dinoflagellates), induces a similar response of upregulation of NOS and oxidative stress, leading to host cell death, when it infects the epithelial cells of the host mosquito *Anopheles stephensi* (Kumar et al., 2004). We therefore hypothesize that ROS is acting as a signal to induce the host cell production of NO during heat stress in symbiotic *A. pallida* (Fig. 5).

Our results indicate that NO is an important mediator of bleaching in *A. pallida* (Fig. 4) and therefore NO could be acting as a cytotoxic compound during bleaching (Lamattina et al., 2003). Incubating anemones with the NO donor SNP resulted in bleaching while co-incubation with the NO scavenger cPTIO decreased the rate of bleaching in both heat-stressed anemones and anemones incubated in SNP. Production of NO during heat stress was assayed after 24 h of exposure to elevated temperatures, but it is likely that production of NO began at an earlier time point after stress to elicit bleaching, given that symbiotic *A. pallida* had already expelled algae within 24 h of exposure to elevated temperatures. We hypothesize that the production of NO could have deleterious effects in conjunction with increased oxidative stress brought about by the effect of elevated temperature on the photosynthetic apparatus of the algae.

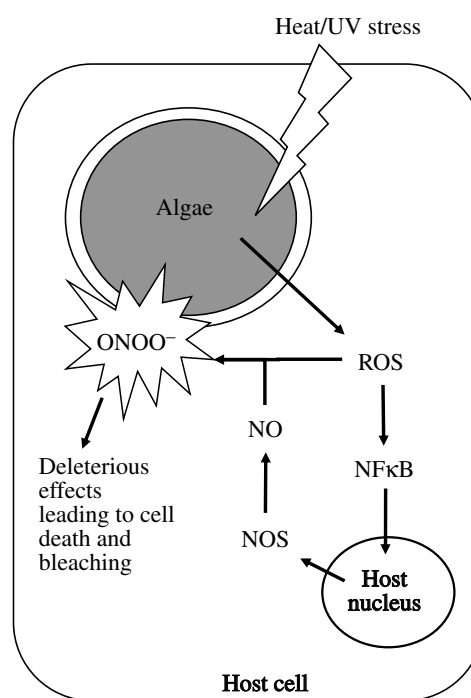


Fig. 5. Proposed model for the role of nitric oxide during cnidarian bleaching. The host cell responds to algal-derived ROS (including superoxide and hydrogen peroxide) by producing NO through signaling leading to the upregulation of NOS. This signaling could involve the transcription factor NF κ B, an important mediator of NOS transcription in other systems. The reaction of superoxide with NO produces the reactive nitrogen species peroxynitrite (ONOO⁻), with additive deleterious effects leading to cell death and bleaching.

NO can react with superoxide to form the reactive nitrogen species peroxynitrite (ONOO⁻), which can act in several deleterious ways. It can (1) undergo decomposition reactions to produce the highly reactive hydroxyl radical (Beckman et al., 1990; Radi et al., 2000); (2) inactivate several steps in the mitochondrial electron transport chain and ATP synthesis, thereby increasing the production of ROS (Radi et al., 2000); (3) inactivate Mn-SOD, leading to a rise in the steady-state levels of ROS and (4) influence homeostasis by increasing the permeability of the mitochondrial membrane (Radi et al., 2000). This last mechanism is critical since it is known to cause the release of potent pro-apoptotic molecules such as cytochrome *c* from mitochondria. We hypothesize that the negative effect of high levels of NO during bleaching is in part due to its conversion to cytotoxic peroxynitrite concomitant with the increase in oxidative stress (Fig. 5).

Some coral-alga associations are more tolerant of hyperthermic bleaching than others (Fitt and Warner, 1995). The observed differences in tolerance to elevated temperature may result from the differential tolerance of the algal strain (phyloptype) present (Buddemeier and Fautin, 1993; Perez et al., 2001; Rowan et al., 1997). Since we do not fully understand the bleaching mechanism, we also do not know how the differential susceptibility of the algae translates mechanistically into differential bleaching. Could differential host production of NO in response to differences in the algal tolerance explain this variation in bleaching? Based on our model, we hypothesize that the differential susceptibility to heat stress of algal photosynthesis also leads to differential levels of oxidative stress and therefore triggers differential production of NO by the host.

The results of this study show that, upon exposure to elevated temperature, symbiotic *A. pallida* produce NO through an NOS-like system and that this process leads to bleaching. We propose that bleaching is an innate animal response to algal dysfunction that, in turn, has been linked to temperature stress. This response is similar to that described in some mammalian systems exposed to pathogens together with oxidative stress and therefore suggests that there is a remarkable degree of conservation of these cellular processes. The cytotoxic effect of NO during bleaching is likely dependent in part on the levels of oxidative stress and the production of damaging reactive nitrogen species such as peroxynitrite (Fig. 5). This hypothesized detrimental interaction between NO and superoxide provides additional insight into the well-described importance of the superoxide-detoxifying enzyme superoxide dismutase in cnidarian-alga symbioses (Richier et al., 2005; Richier et al., 2003).

Another example of conservation of cellular mechanisms that spans large taxonomic distances is the production of NO in *A. pallida* upon exposure to LPS. This mechanism may explain bleaching in corals exposed to *Vibrio* sp. and bacterial SOD as a virulence factor in that process (Ben-Haim et al., 2003). The regulatory role of NO as a function of oxidative stress may also explain some of the bleaching variability observed in both the laboratory and the field linked to the

differential tolerance of algal photosynthesis to elevated temperatures. Nitric oxide is emerging as an important regulator in both parasitic and mutualistic symbioses and this illustrates the importance of understanding the innate immune response mediating host-symbiont interactions in these systems.

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