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Role of the Sphingosine Rheostat in the Regulation of Cnidarian-Dinoflagellate Symbioses

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Abstract. The symbiosis between host cnidarians, such as corals and anemones, and their dinoflagellate symbionts is regulated by largely undescribed mechanisms that stabilize the symbiosis during normal conditions but lead to symbiosis breakdown, or cnidarian bleaching, during stress. Previous transcriptomic studies identified the sphingosine rheostat as a putative symbiosis regulatory pathway. The sphingosine rheostat, which includes the sphingolipids sphingosine (Sph) and sphingosine 1-phosphate (S1P), is a key homeostatic cell regulatory pathway known to function in cell fate and immunity in animals. This study explores the role of sphingosine rheostat components in the stability of the symbiotic partnership. The anemone *Aiptasia pallida*, host to the dinoflagellate *Symbiodinium* sp., was used to test the hypothesis that S1P promotes symbiosis stability whereas Sph increases bleaching induced by heat stress. Anemones pre-incubated in exogenous S1P and FTY720, a synthetic S1P analog, were partially rescued from heat-stress-induced bleaching. In addition, they displayed a decrease in caspase activity, a measure of apoptosis, compared to controls. In contrast, when anemones were pre-incubated with Sph, both bleaching and caspase activity increased compared to untreated, heat-stressed controls. These data suggest that the sphingosine rheostat may play a role in the balance between stability and dysfunction in cnidarian-dinoflagellate symbioses.

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Abbreviations: DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DAN, 2,3-diaminonaphtalene; FASW, filtered artificial seawater; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NO, nitric oxide; S1P, sphingosine 1-phosphate; Sph, D-sphingosine; SPPase, sphingosine 1-phosphate phosphatase; VO, vehicle only (0.1% v/v dimethyl sulfoxide).

Introduction

The regulation of mutualistic endosymbioses between cnidarians and dinoflagellates, such as those that characterize coral reef ecosystems, is complex and involves mechanisms of symbiosis onset, maintenance, and breakdown. Much of the regulatory communication between the partners that results in the development of a stable association is just beginning to be discovered. Studies to date point toward complex, multistep cellular interactions between the partners, including mechanisms of invasion by the symbiont (members of the genus *Symbiodinium*) and mechanisms of recognition by host-cell immunity that together result in host tolerance of the symbionts, ultimately leading to stability of the partnership (Wood-Charlson *et al.*, 2006; Dunn and Weis, 2009; Weis and Allemand, 2009; Kvennefors *et al.*, 2010). Likewise, mechanisms of symbiosis dysfunction and breakdown that lead to cnidarian bleaching are only partially understood. Symbiosis collapse occurs as a result of environmental stress such as global warming. It is initiated by high levels of reactive oxygen species from damaged symbionts (Lesser, 2006; Venn *et al.*, 2008) that are now thought to trigger innate immunity signaling cascades in the host (Lesser and Farrell, 2004; Perez and Weis, 2006; Weis, 2008). This host response ultimately causes loss of symbionts by several mechanisms, including host-cell apoptosis (Richier *et al.*, 2006; Dunn *et al.*, 2007; Weis, 2008). Gene discovery studies in symbiotic corals and sea anemones have begun to reveal clues about the regulatory workings of symbiosis health and dysfunction. One such microarray study (Rodriguez-Lanetty *et al.*, 2006) compared partial transcriptomes of symbiotic and nonsymbiotic (aposymbiotic) specimens of the sea anemone *Anthopleura elegantissima* to identify genes differentially expressed as a function of the symbiotic state. Among the genes that were downregulated in host tissues in the symbiotic state was a homolog of the enzyme sphingosine 1-phosphate phospha-

Table 1

Sphingosine pathway components identified in cnidarians

Protein	Cnidarian species	NCBI accession/ Protein ID JGI	BLAST hit
S1P phosphatase (SPPase)	*Anthopleura elegantissima*	ABD04172	XP_001343219 sphingosine-1-phosphate phosphatase 1 [*Danio rerio*]
	Nematostella vectensis	XP_001636092, JGI ID:13329, 182836, 145844	XP_001343219 sphingosine-1-phosphate phosphatase 1 [*Danio rerio*]
	Nematostella vectensis	XP_001621147	XM_001343183.3 sphingosine-1-phosphate phosphatase 1 [*Danio rerio*]
Sphingosine kinase	*Nematostella vectensis*	JGI ID:108592	NP_001136073 sphingosine kinase 1 [*Homo sapiens*]

tase (SPPase), a key participant in the pathway regulating the balance of signaling sphingolipids.

The signaling sphingolipids sphingosine (Sph) and sphingosine 1-phospate (S1P) participate in a myriad of cellular and physiological processes affecting cell homeostasis and function, including differentiation, immunity, and the determination of cell fate (*i.e.,* survival or death) (Spiegel and Milstien, 2000). These are ancient signaling molecules as demonstrated by their presence in yeast, plants, and metazoans (Spiegel and Milstien, 2000; Oskouian and Saba, 2004). The balance between Sph and S1P can be regulated by the so-called sphingosine rheostat, whereby Sph is phosphorylated to S1P by sphingosine kinase, and conversely S1P is dephosphorylated to Sph by SPPase. The rheostat can be controlled in several ways, including by regulating expression of sphingosine kinase and SPPase. Elevated levels of S1P promote cell survival and proliferation, whereas elevated Sph leads to apoptotic cell death. Both act through numerous pleiotropic downstream signaling cascades (Spiegel and Milstien, 2000). S1P initiates signaling cascades both endogenously and exogenously through mechanisms that are in some cases still not well understood (Rosen and Goetzl, 2005). Among the best-studied S1P signaling pathways in animals is the binding of exogenous S1P to several S1P membrane receptors, members of the G-protein-coupled receptor gene family (Rosen and Goetzl, 2005; Rivera *et al.*, 2008). In addition to the SPPase homolog from *A. elegantissima*, bioinformatic searches of the genome of the anemone *Nematostella vectensis* show evidence of the sphingosine rheostat in cnidarians (Table 1), and numerous G-protein-coupled receptors that could be candidates for binding S1P as a ligand.

Identification of differentially expressed SPPase in symbiotic anemones led us to hypothesize that sphingolipids are involved in the regulation of cnidarian-dinoflagellate symbioses (Rodriguez-Lanetty *et al.*, 2006). In this study we set out to test this idea by using the model symbiotic anemone *Aiptasia pallida* (Gosse, 1858). We specifically hypothesized that (1) S1P suppresses a host innate immune reponse and promotes host-cell survival and therefore symbiosis stability, and (2) Sph promotes an immune response and elevates heat-stress-induced bleaching. We measured levels of bleaching in anemones treated with exogenous S1P and its synthetic analog FTY720, or exogenous Sph. In addition, we measured both caspase activity and nitric oxide (NO) production in heat-stressed anemones in the presence of exogenous S1P, FTY720, or Sph to see if apoptotic activity and innate immune responses were likewise respectively reduced or increased compared to levels in heat-stressed animals in the absence of treatment. The data suggest that sphingolipids are participating in the regulation of the cnidarian-dinoflagellate partnership.

Materials and Methods

Anemone cultures

Specimens of *Aiptasia pallida* were collected in the Florida Keys and maintained in an aquarium of artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) that was kept at room temperature on a photoperiod of 12 h light:12 h dark with an irradiance of 70 μmol quanta m^{-2} s^{-1}. Aposymbiotic anemones were kept in the same conditions, but in total darkness. Four weeks prior to an experiment, anemones were transferred into bowls containing 0.22 μm of filtered artificial seawater (FASW), placed in an incubator set at 25 °C, and kept in their respective light regimes. Anemones were fed once a week with freshly hatched brine shrimp. One week before an experiment, animals were starved and transferred to 24-well plates (~5 ml per well).

Hyperthermic stress conditions

The influence of the sphingosine rheostat on heat-stress-induced bleaching was studied using symbiotic anemones incubated for 2 h in either vehicle only (VO; 0.1% v/v DMSO), 10 μmol l^{-1} S1P or 1 μmol l^{-1} FTY720, a synthetic sphingosine receptor (SPR) agonist (Cayman Chemical, Ann Arbor, MI) or 1 μmol l^{-1} Sph (Sigma, St. Louis, MO) ($n = 3$ animals per treatment), before being subjected to a 24-h hyperthermic stress at 33 °C , a stress known to elicit bleaching (Dunn *et al.*, 2004; Perez and Weis, 2006).

Table 2

Summary of probes used in experiments and their excitation and emission wavelengths

Labeled structure, activity or molecule	Dye	Excitation wavelength (nm)	Emission wavelength (nm)
NO	15 μmol l^{-1} DAF-FM	488	510–530
Caspase activity	50 μmol l^{-1} Rhodamine110	488	505–530
Host nuclei	1.5 μmol l^{-1} DAPI	405	420–480
Dinoflagellates	None (detection of chlorophyll autofluorescence)	543	600–700

The anemones were held in their respective pharmacological treatment throughout the experiment. Loss of symbionts from host tissues (bleaching) was quantified using methods published previously (Perez and Weis, 2006; Dunn *et al.*, 2007). Percent bleaching was calculated as the number of expelled symbionts/(expelled symbionts + number of symbionts *in hospite*) × 100. In another set of heat-stress experiments designed to measure an innate immune response, anemones were pretreated for 4 h with VO or 0.01, 0.1, 1.0, or 10 μmol l^{-1} of either S1P or FTY720 or 0.2, 1, or 5 μmol l^{-1} Sph before being heat-stressed as described above. Immune response was measured by fluorometrically quantifying caspase activity in host tissues or NO in anemone homogenates (described below).

Lipopolysaccharide-induced immune response

To explore the influence of activation of the sphingosine pathway on cnidarian immune status, aposymbiotic anemones were incubated for 4 h in FASW supplemented with VO or 1 μmol l^{-1} of either S1P, FTY720, or Sph. Following this, to elicit an immune response, anemones were incubated overnight in 1 μg/ml of lipopolysaccharide (LPS) (Sigma, St Louis, MO) or VO. Immune response was measured by quantifying NO fluorometrically in host tissue or in anemone homogenates by confocal microscopy or DAN assay respectively (described below).

Confocal microscopy to measure caspase activity and nitric oxide production in host tissues

Confocal microscopy on whole anemones was used to visualize NO, caspase activity, and host nuclei by using specific fluorescent dyes solubilized in relaxing solution (1:1, 370 mmol l^{-1} $MgCl_2$:FASW). Anemones were incubated for 30 min in the dark and then rinsed twice with relaxing solution before image acquisition. Images were acquired with a Zeiss LSM 510 Meta microscope with a 40×/0.8 water objective lens and a working distance of 0.8–3.2 mm; the excitation and emission wavelengths used are detailed in Table 2. Mean fluorescence intensity (MFI) in the gastrodermal tissue of anemone tentacles was quantified using the LSM 5 software.

To measure and visualize production of NO, animals were loaded with 15 μmol l^{-1} 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, Molecular Probes, Eugene, OR) (Nagano and Yoshimura, 2002; Perez and Weis, 2006). To detect host-cell caspase activity, 50 μmol l^{-1} of the fluoroprobe Rhodamine 110 aspartic acid caspase substrate (Molecular Probes, Eugene, OR), dissolved in 0.2% DMSO, was used. Upon enzymatic cleavage by caspases, the nonfluorescent bisamide substrate is converted to the fluorescent Rhodamine 110. In the experiments examining caspase activity, to aid in specimen orientation, animals were also loaded with 1.5 μmol l^{-1} of the fluorescent DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, St Louis, MO) to visualize host nuclei. *Symbiodinium* present in symbiotic anemones were visualized by detecting chlorophyll autofluorescence.

NO_2^- quantification as a measurement for nitric oxide (DAN method)

In some experiments, instead of using confocal microscopy, NO production in homogenized animals was quantified fluorometrically using 2,3-diaminonaphtalene (DAN) (Dojindo, Gaithersburg, MD) with methods modified from Nussler *et al.* (2006). Secreted NO is very unstable and reacts almost instantly with oxygen to form NO_2^-. NO_2^- quantification is therefore an indirect method used to evaluate NO production. DAN reacts with NO_2^- in acidic conditions to produce fluorescent naphthalenetriazole. Briefly, after experimental treatment, anemones were rinsed in 100 ml of 370 mmol l^{-1} $MgCl_2$ just before being flash-frozen in liquid nitrogen and stored at −80 °C. Anemones were thawed on ice and homogenized in 340 μl of deionized water at 4 °C. The solution was centrifuged at 14,000 × *g* for 10 min at 4 °C. Five microliter of the supernatant was used for protein determination (Bradford assay). The remaining supernatant was filtered through a prewashed (3 times in 500 μl of deionized water) Centricon column (10 kDa cutoff). A standard curve was constructed using 10,000, 5,000, 2,500, 1,250, 625, 312, and 156 nmol l^{-1} $NaNO_2$. One hundred fifty microliter of standard or sample was added to wells of a 96-well black-bottom plate (Nunc Black 96F polysorp; Fisher Scientific, Rochester, NY) and 75 μl of a DAN solution at 158 μmol l^{-1} /HCl 0.62 N was added to each well. The plate was mixed for 10 s and incubated at 28 °C in the dark for 10 min before adding 35 μl of 2 N NaOH. Finally, the DAN NO_2^--dependent fluorescence at 410 nm was quantified after excitation at 365 nm in a fluorescent platereader. Values were expressed as μmol l^{-1} NO_2^- per microgram of protein.

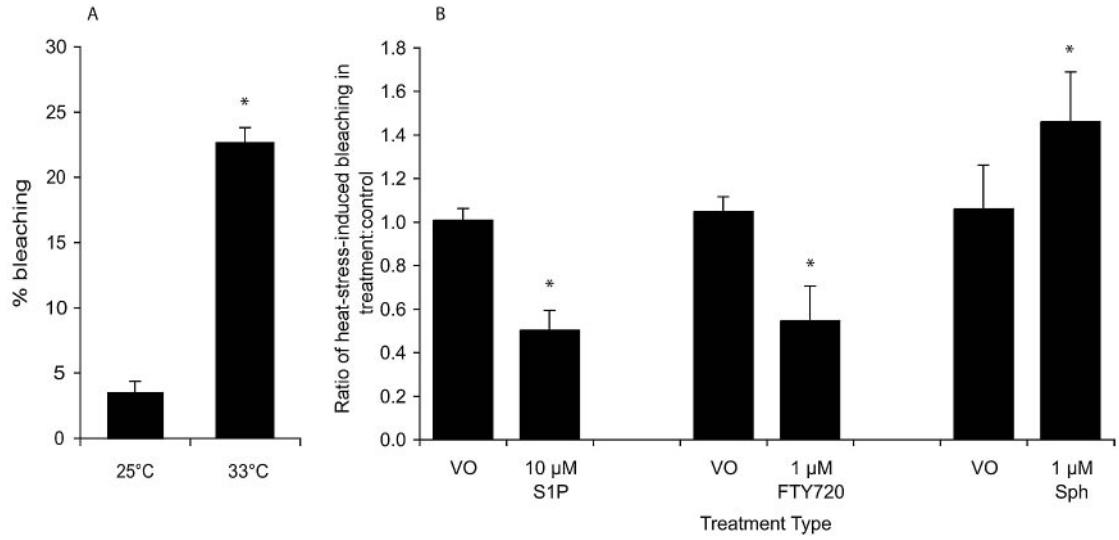

Figure 1. Addition of exogenous sphingolipids modifies the bleaching response in heat-stressed *Aiptasia pallida.* (A) Bleaching (percent of symbionts lost) was quantified in anemones incubated for 24 h at ambient (25 °C) or elevated (33 °C) temperature. Symbiont loss was quantified after an additional 24 h at ambient temperature. (Bars represent means + SD; $n = 3$ anemones per treatment; t test, $^*P < 0.05$.) (B) For experimental treatments, prior to elevated temperature exposure, anemones were pre-incubated for 2 h in DMSO vehicle only (VO), 10 μmol l^{-1} S1P, 1 μmol l^{-1} FTY720, or 1 μmol l^{-1} Sph. Results are expressed as ratios of the amount of heat-stress-induced bleaching in treatments: nontreatment controls. (Bars represent means + SD; $n = 3$ anemones per treatment; t test, $^*P < 0.05$.)

Results

After 24 h of heat stress at 33 °C, anemones lost more symbionts (22.67% ± 1.15% lost) than those kept at 25 °C (3.6% ± 0.78%) (Fig. 1A). These data are consistent with many other published reports of heat-stress-induced bleaching in *Aiptasia pallida* (Dunn *et al.*, 2004, 2007; Perez and Weis, 2006). To test the hypothesis that the sphingosine rheostat plays a role in symbiosis stability, symbiotic anemones were incubated in DMSO vehicle only (VO), the exogenous SPR agonists S1P and FTY720 (10 μmol l^{-1} and 1 μmol l^{-1} respectively), or exogenous Sph (1 μmol l^{-1}) before being heat-stressed. To enable direct comparison between experiments, values in Figure 1B are ratios of heat-stress-induced bleaching in treatments: untreated controls. All VO-incubated animals exhibited rates of bleaching similar to those of untreated animals (ratios of ~1). Anemones incubated in exogenous SPR agonists exhibited less bleaching than VO animals, with ratios of 0.50 ± 0.09 and 0.55 ± 0.16 for S1P and FTY720 treatments respectively. These decreases respectively corresponded to 50% and 48% decreases in bleaching compared to VO animals. In contrast, animals pretreated in Sph had an enhanced ratio of 1.46 ± 0.23—an increase in bleaching of 38% over VO animals.

Next we were interested in examining the cellular pathways that could result in the rescue of a bleaching response in the presence of S1P. High levels of S1P have been shown to favor cell survival and proliferation, whereas high sphingosine promotes apoptotic cell death (Spiegel and Milstien, 2000). Despite evidence that cnidarian bleaching is caused by a complexity of cellular mechanisms that depend on the nature and severity of the stressor, host-cell apoptosis is believed to be one of the central players in the response. To test the hypothesis that the sphingosine rheostat plays a role in host-cell homeostatic decisions to either survive and proliferate or to die, host-cell apoptosis was measured in heat-stressed anemones in the presence and absence of exogenous SPR agonists or Sph, using fluorescent Rhodamine 110, a measure of caspase activity (Fig. 2). Untreated anemones at ambient temperature (25 °C) had a relatively consistent mean caspase activity among all three experiments (ranging from 10.1 ± 1.0 up to 12.8 ± 3.7 MFI) (Fig. 2A–C). Caspase activity in VO heat-stressed animals had consistently elevated mean activity of 25.23 ± 0.81 MFI among all three experiments (Fig. 2A–C). MFI values trended down with increasing S1P concentrations; however, they were significantly lower than VO heat-stressed values only at 0.1 μmol l^{-1} S1P, in the middle of the concentration series with a mean value of 6.5 ± 0.2 (Fig. 2A). Caspase activity in anemones exposed to a range of FTY720 concentrations decreased from the VO heat-stressed value in a dose-dependent manner, with the differ-

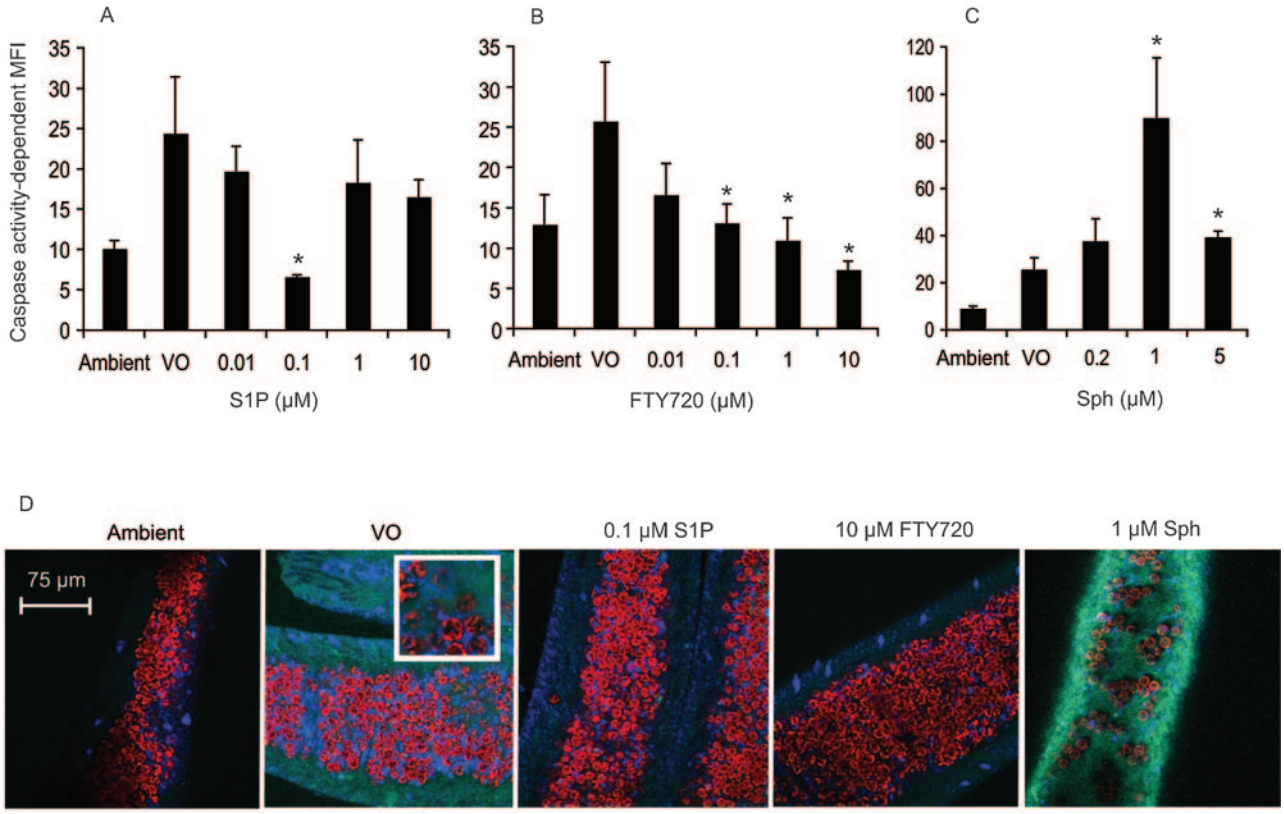

Figure 2. Addition of exogenous sphingolipids modifies heat-stress-induced caspase activity in *Aiptasia pallida.* Symbiotic anemones were incubated in a range of (A) S1P, (B) FTY720, (C) Sph concentrations for 2 h before being subjected to a heat stress at 33 °C for 24 h. The effect of agonists on caspase activity was measured using Rhodamine 110 fluorescence and confocal microscopy. Caspase activity decreased in a dose-dependent manner for FTY720-treated animals, but not for those incubated in S1P. In contrast, Sph treatment increased caspase activity in heat-stressed anemones. (Bars represent means + SD; $n = 3$ anemones per treatment; ANOVA *post hoc* Bonferroni, $^*P < 0.05$.) (D) Representative confocal images of non-heat-stressed (ambient) and heat-stressed animals pretreated with DMSO vehicle only (VO), 0.1 μmol l^{-1} S1P, 10 μmol l^{-1} FTY720, or 1 μmol l^{-1} Sph. Inset: higher magnification of anemone tentacle cross-section. Green = caspase-specfic fluorescence, blue = host-cell nuclei, red = symbiont autofluorescence. Caspase-specific fluorescence, present in control animals, is not evident in agonist-treated animals.

ences being significant from VO MFI starting at 0.1 μmol l^{-1} and ending with the lowest value of 7.2 ± 1.1 MFI at 10 μmol l^{-1} FTY720 (Fig. 2B). Although the animals responded differently to the two agonists, in both cases the presence of S1P agonist decreased host-cell caspase activity. In contrast, pre-incubation of anemones with Sph before heat stress enhanced the induction of caspase activity in a dose-dependent manner, with a peak of significant activation occurring at 1 μmol l^{-1} Sph with a value of 90.0 ± 25.4 MFI (Fig. 2C). Caspase activity in confocal images was evident as green staining in epidermis and in the gastrodermis housing resident symbionts, as shown in heat-stressed animals in VO and Sph but not in animals in S1P, FTY720, or in untreated animals incubated at ambient temperature (Fig. 2D)

S1P is a major signaler in the control of both innate and adaptive immunity in vertebrates (Rivera *et al.*, 2008). We therefore set out to test a third hypothesis that the sphingosine rheostat is participating in the innate immunity cascade of the host cell to maintain a stable symbiosis. Previous work points toward a role for innate immunity in cnidarian bleaching (Lesser and Farrell, 2004; Perez and Weis, 2006; Safavi-Hemami *et al.*, 2010). NO is a cytotoxic signaling molecule that functions in innate immunity in a variety of organisms. NO levels are very high in heat-stressed anemones, and addition of exogenous NO can cause anemones to bleach at ambient temperature (Perez and Weis, 2006). These findings led to the idea that heat stress causes a cellular response related to an innate immune response that results in elimination of symbionts from hosts.

The testing of this third hypothesis was divided into two steps. First, a link between the sphingosine rheostat and innate immunity in anemones was investigated by culturing aposymbiotic anemones with the classic immune elicitor

lipopolysaccharide (LPS). Because LPS induces almost no production of NO in symbiotic anemones (Detournay and Weis, unpubl. data), we stimulated aposymbiotic anemones in this series of experiments. As reported previously (Perez and Weis, 2006), aposymbiotic anemones produced a strong NO signal in response to incubation with LPS compared to LPS– controls (Fig. 3 A, B). In addition, aposymbiotic anemones were pre-incubated with either VO, 1 μmol l^{-1} S1P, FTY720, or Sph prior to incubation in LPS and then measured for NO signal with the NO probe DAF-FM DA or by the DAN assay (see Materials and Methods). To enable a comparison between different experiments, results were expressed as percent of mean values obtained with LPS-incubated, no-treatment controls. NO signal significantly decreased with the addition of both S1P and FTY720 compared to VO animals (Fig. 3C). There was, however, no difference in NO signal between Sph- and VO-treated animals. These findings indicate that, at least, exogenous SPR agonists are modulating the immune response to LPS.

With data to suggest that S1P can play a role in an immune response in anemones, we next set out to test for its role in modulating an immune response during symbiosis. Symbiotic anemones were pre-incubated with SPR agonists or Sph concentrations known to have a suppressive effect on NO production, prior to subjecting animals to an elevated temperature of 33 °C for 24 h. Thereafter, the NO signal was measured by using confocal microscopy or the DAN assay. Even though we were able to observe heat-stress-induced NO secretion in symbiotic anemones (Fig. 4A, B), to our surprise, the addition of neither SPR agonists nor Sph changed the NO signal in heat-stressed anemones relative to VO treatments (Fig. 4C). This suggests that, although S1P can affect an immune response in anemones, changes in the sphingosine rheostat do not affect elevated NO levels observed in heat-stressed animals.

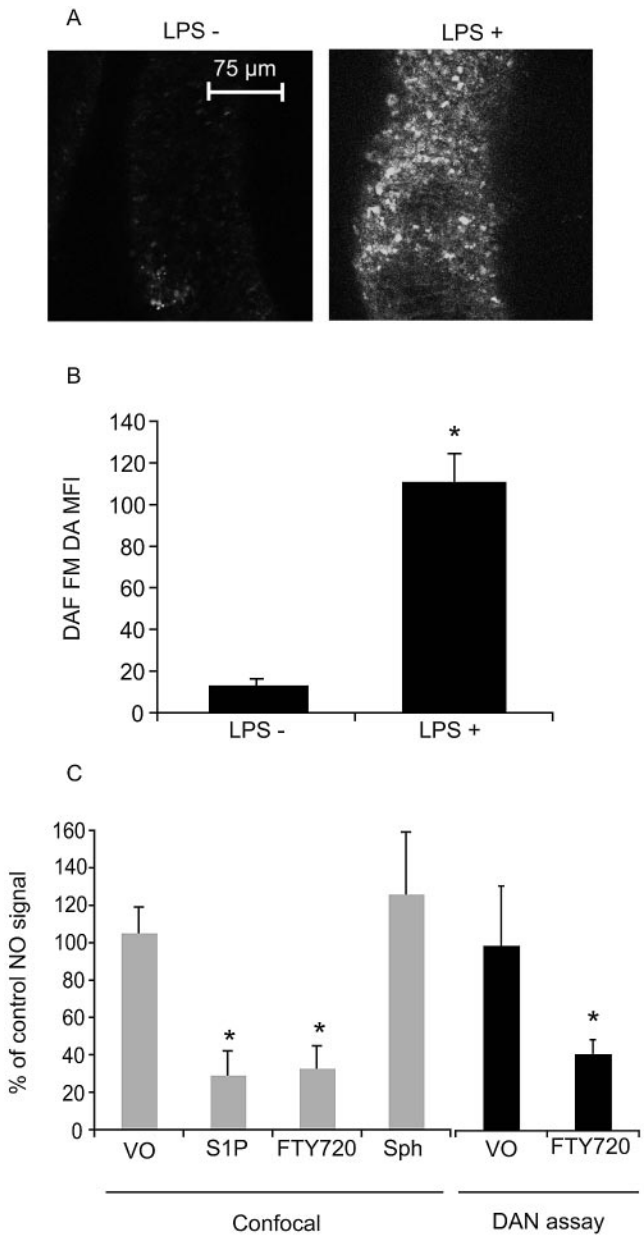

Figure 3. The sphingosine rheostat influences lipopolysaccharide (LPS)-induced NO production in aposymbiotic *Aiptasia pallida.* (A) Confocal images of DAF-FM-loaded tentacles of anemones nontreated (LPS–) or immune-stimulated overnight in 1 μg/ml LPS (LPS+). (B) Quantification of DAF-FM fluorescence in response to LPS stimulation compared to untreated anemones (LPS–). Results are expressed as NO-specific signal in mean fluorescence intensity. (Bars represent means + SD; $n = 3$; 2-tailed t test , $^*P < 0.05$.) (C) Anemones were incubated in 1 μmol l^{-1} SPR agonists or Sph for 2 h before being LPS-stimulated. Results obtained by confocal analysis or with the DAN assay are expressed as percent of mean NO signal obtained with LPS only. (Bars represent means + SD; $n = 3$ LPS-stimulated anemones per treatment; 2-tailed t test , $^*P < 0.05$).

Discussion

Pushing the sphingosine rheostat towards S1P with addition of exogenous S1P or the synthetic analog FTY720 preserved symbiosis stability and discouraged dysfunction. The opposite effect was obtained by treatment of anemones with Sph (Fig. 1). Together, these findings indicate that the rheostat is participating in the regulation of symbiosis stability.

Addition of exogenous SPR agonists decreased caspase activity in anemones subjected to a hyperthermic stress, while exogenous addition of Sph increased caspase activity (Fig. 2). This suggests that the sphingosine rheostat could play a role in determining cell fate and that during bleaching the rheostat could push host cells towards apoptosis. Understanding the significance of the different responses to S1P and FTY720 dose responses requires further investigation and a deeper understanding of the specific cascades that S1P levels trigger. For example, in human cells the anti-apoptotic effects of exogenous S1P are S1P-receptor-dependent and require submicromolar concentrations of agonist

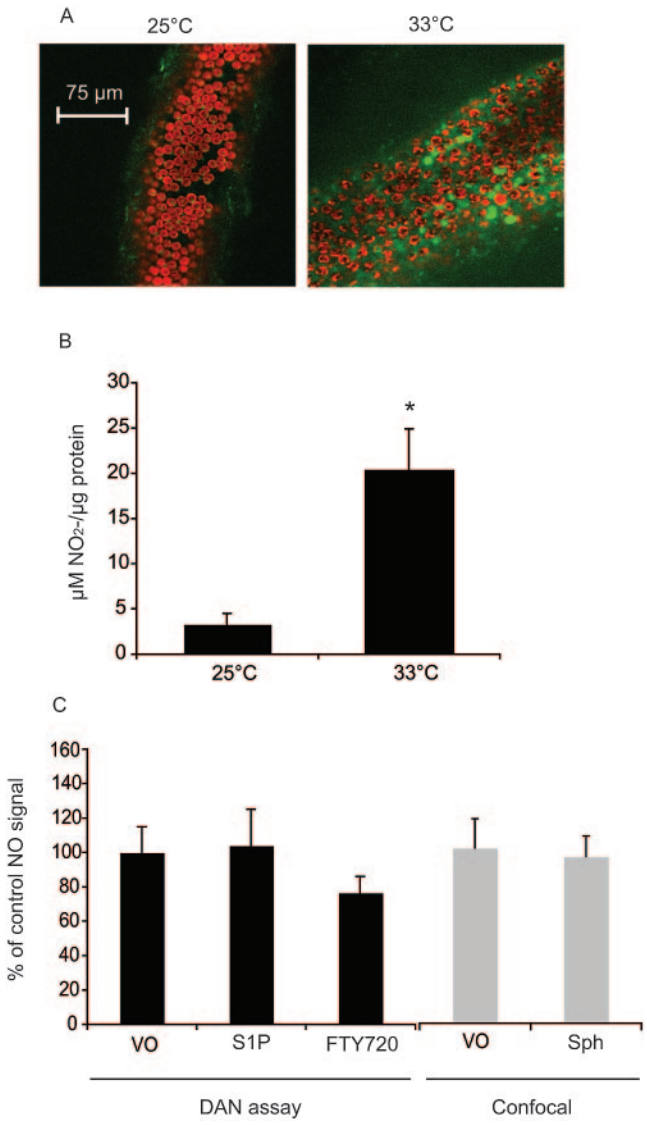

Figure 4. Addition of exogenous sphingolipids does not affect heat-stress-induced NO production in *Aiptasia pallida.* (A) Confocal images of DAF-FM-loaded tentacles of anemones at ambient (25 °C) or elevated (33 °C) temperature. (B) DAN assay quantification of NO secretion in homogenized symbiotic anemones incubated at ambient (25 °C) or elevated (33 °C) temperature. Results are expressed as μmol l^{-1} NO_2^- per μg protein. (Bars represent means + SD; $n = 3$; 2-tailed t test, $^*P < 0.05$) (C) DAN assay or confocal analyses were used to test the effect of SPR agonists or Sph on heat-stress-induced NO production in anemones. Symbiotic anemones were pretreated in 1 μmol l^{-1} SPR agonists or Sph for 2 h before being heat-stressed for 24 h at 33 °C. Results are expressed as percent of mean NO signal obtained with heat stress only. (Bars represent means + SD; $n = 3$ heat-stressed anemones per treatment.) No significant difference was observed between treatments (2-tailed t test, $^*P > 0.05$).

(Davaille *et al.*, 2000). In addition, other reports have shown that only the phosphorylated form of FTY720 binds S1P receptors (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002). Since we used non-phosphorylated FTY720 in our experiments, it is possible that, depending on the rate of phosphorylation of FTY720, we obtained results that differed from those obtained with S1P. We observed that 10 μmol l^{-1} of S1P had a weak effect on decreasing caspase activity (Fig. 2A) but a strong effect of rescuing bleaching (Fig. 1). First, this suggests that the rescue effect of S1P on bleaching would be an increased if we had chosen a submicromolar pre-incubation concentration. Second, it suggests that the sphingosine rheostat could be acting in ways in addition to promotion of cell survival and blocking of apoptosis to maintain a stable symbiosis.

High levels of SPR agonist modulate an anemone innate immune response characterized by LPS-induced NO production (Fig. 3C). This suggests that the sphingosine rheostat is participating in cnidarian host-cell innate immunity. However, exogenous addition of SPR agonists had no effect on heat-stress-induced production of NO by the host (Fig. 4C). Similarly, we did not observe any effect of Sph pretreatment on LPS– (Fig. 3C) or heat-stress-induced (Fig. 4C) NO secretion. This suggests that NO secretion is not dependent on the sphingosine rheostat and that the rescuing effect of SPR agonists on heat-stress-induced bleaching is not correlated to a modulation of NO production. Indeed, NO production can be induced by different mechanisms. LPS induction of NO occurs through toll-like receptor (TLR) (Beutler *et al.*, 2003); however, reactive oxygen species can also induce NO, and this is the mechanism that is likely to underlie NO production during heat stress (Perez and Weis, 2006; Weis, 2008). In this context, S1P has been shown to selectively attenuate TLR signaling in murine macrophages, thus preventing inflammatory activation (Duenas *et al.*, 2008). The knowledge that the TLR pathway is present in cnidarians (Miller *et al.*, 2007) suggests that the SPR agonist effects observed on LPS-induced NO production (Fig. 3) could be due to a mechanism comparable to that described in mice.

In summary, we can add some detail to the testable model proposed by Rodriguez-Lanetty and coworkers (Rodriguez-Lanetty *et al.,* 2006), in which the sphingosine rheostat plays a role in the maintenance of symbiosis (Fig. 5). An invading organism such as a dinoflagellate symbiont is recognized by the host by means of one or more immunogenic signals (for example, glycans). An innate immune cascade is initiated that leads to the activation of effector pathways, including apoptosis, aimed at eliminating the foreigner unless a second tolerogenic signal acts to push the sphingosine rheostat toward S1P by downregulating SPPase. The resulting accumulation of S1P leads to the survival of the host cell by inhibiting apoptosis. The balance of these pathways is somehow disturbed by heat stress. One scenario is that heat stress leads to alteration of the sphingosine rheostat—for example, by increasing SPPase production (Sawyer and Muscatine, 2001), thereby decreasing pro-

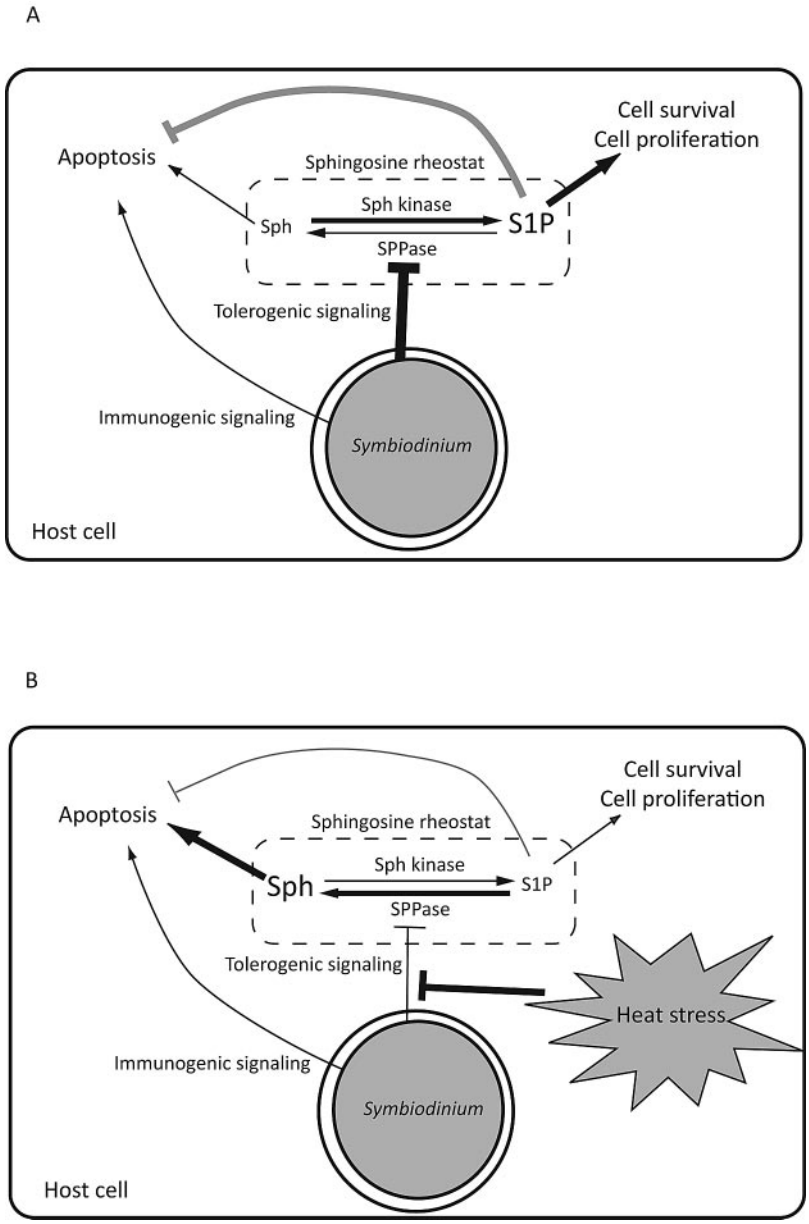

Figure 5. Model describing the role of the sphingosine rheostat in the stability of cnidarian-dinoflagellate symbioses. (A) Host cnidarian cell symbiotic with *Symbiodinium* under stable conditions. Immunogenic signals by the symbionts promote host-cell apoptosis unless tolerogenic signals act on the sphingosine rheostat by decreasing SPPase activity, which in turn increases S1P levels, thereby promoting cell survival and blocking apoptosis. (B) Host cnidarian cell symbiotic with *Symbiodinium* undergoing heat stress. Heat stress somehow disturbs the balance of the sphingosine rheostat. Heat stress blocks the tolerance signal, which lowers SPPase activity. This favors the buildup of sphingosine, which in turn promotes apoptosis. *Abbreviations:* Sph, sphingosine; S1P, sphingosine 1-phosphate; SPPase, sphingosine 1-phosphate phosphatase.

duction of S1P, which in turn promotes apoptosis and bleaching. Much work needs to be done to confirm a role for the sphingosine rheostat in symbiosis stability. This work includes measurement of levels of S1P and rheostat enzyme activity and identification of putatitve S1P receptors and downstream effector pathways initiated by S1P.

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Literature Cited

Beutler, B., K. Hoebe, X. Du, and R. J. Ulevitch. 2003. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukoc. Biol.* **74:** 479–485.

Brinkmann, V., M. D. Davis, C. E. Heise, R. Albert, S. Cottens, R. Hof, C. Bruns, E. Prieschl, T. Baumruker, P. Hiestand, *et al.* 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.* **277:** 21453–21457.

Davaille, J., C. Gallois, A. Habib, L. Li, A. Mallat, J. Tao, T. Levade, and S. Lotersztajn. 2000. Antiproliferative properties of sphingosine 1-phosphate in human hepatic myofibroblasts. A cyclooxygenase-2 mediated pathway. *J. Biol. Chem.* **275:** 34628–34633.

Duenas, A. I., M. Aceves, I. Fernandez-Pisonero, C. Gomez, A. Orduna, M. S. Crespo, and C. Garcia-Rodriguez. 2008. Selective attenuation of Toll-like receptor 2 signalling may explain the atheroprotective effect of sphingosine 1-phosphate. *Cardiovasc. Res.* **79:** 537–544.

Dunn, S. R., and V. M. Weis. 2009. Apoptosis as a post-phagocytic winnowing mechanism in a coral-dinoflagellate mutualism. *Environ. Microbiol.* **11:** 268–276.

Dunn, S. R., J. C. Thomason, M. D. Le Tissier, and J. C. Bythell. 2004. Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration. *Cell Death Differ.* **11:** 1213–1222.

Dunn, S. R., C. E. Schnitzler, and V. M. Weis. 2007. Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarian bleaching: every which way you lose. *Proc. R. Soc. B. Biol. Sci.* **274:** 3079–3085.

Kvennefors, E. C., W. Leggat, C. C. Kerr, T. D. Ainsworth, O. Hoegh-Guldberg, and A. C. Barnes. 2010. Analysis of evolutionarily conserved innate immune components in coral links immunity and symbiosis. *Dev. Comp. Immunol*. **34:** 1219–1229.

Lesser, M. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.* **68:** 253–278.

Lesser, M., and J. Farrell. 2004. Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. *Coral Reefs* **23:** 367–377.

Mandala, S., R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G. J. Shei, D. Card, C. Keohane, *et al.* 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296:** 346–349.

Miller, D. J., G. Hemmrich, E. E. Ball, D. C. Hayward, K. Khalturin, N. Funayama, K. Agata, and T. C. Bosch. 2007. The innate immune repertoire in cnidaria—ancestral complexity and stochastic gene loss. *Genome Biol.* **8:** R59.

Nagano, T., and T. Yoshimura. 2002. Bioimaging of nitric oxide. *Chem. Rev.* **102:** 1235–1270.

Nussler, A. K., M. Glanemann, A. Schirmeier, L. Liu, and N. C. Nussler. 2006. Fluorometric measurement of nitrite/nitrate by 2,3-diaminonaphthalene. *Nat. Protoc.* **1:** 2223–2226.

Oskouian, B., and J. D. Saba. 2004. Death and taxis: what non-mammalian models tell us about sphingosine-1-phosphate. *Semin. Cell Dev. Biol.* **15:** 529–540.

Perez, S., and V. Weis. 2006. Nitric oxide and cnidarian bleaching: an eviction notice mediates breakdown of a symbiosis. *J. Exp. Biol.* **209:** 2804–2810.

Richier, S., C. Sabourault, J. Courtiade, N. Zucchini, D. Allemand, and P. Furla. 2006. Oxidative stress and apoptotic events during thermal stress in the symbiotic sea anemone, *Anemonia viridis. FEBS J.* **273:** 4186–4198.

Rivera, J., R. L. Proia, and A. Olivera. 2008. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat. Rev. Immunol.* **8:** 753–763.

Rodriguez-Lanetty, M., W. S. Phillips, and V. M. Weis. 2006. Transcriptome analysis of a cnidarian-dinoflagellate mutualism reveals complex modulation of host gene expression. *BMC Genomics* **7:** 23.

Rosen, H., and E. J. Goetzl. 2005. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat. Rev. Immunol.* **5:** 560–570.

Safavi-Hemami, H., N. D. Young, J. Doyle, L. Llewellyn, and A. Klueter. 2010. Characterisation of nitric oxide synthase in three cnidarian-dinoflagellate symbioses. *PLoS One* **5:** e10379.

Sawyer, S. J., and L. Muscatine. 2001. Cellular mechanisms underlying temperature-induced bleaching in the tropical sea anemone *Aiptasia pulchella. J. Exp. Biol.* **204:** 3443–3456.

Spiegel, S., and S. Milstien. 2000. Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476:** 55–57.

Venn, A. A., J. E. Loram, and A. E. Douglas. 2008. Photosynthetic symbioses in animals. *J. Exp. Bot.* **59:** 1069–1080.

Weis, V. M. 2008. Cellular mechanisms of cnidarian bleaching: stress causes the collapse of symbiosis. *J. Exp. Biol.* **211:** 3059–3066.

Weis, V. M., and D. Allemand. 2009. What determines coral health? *Science* **324:** 1153–1155.

Wood-Charlson, E. M., L. L. Hollingsworth, D. A. Krupp, and V. M. Weis. 2006. Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cell. Microbiol.* **8:** 1985–1993.