

SHORT COMMUNICATION

Nitric oxide and coral bleaching: is peroxynitrite generation required for symbiosis collapse?

Thomas D. Hawkins and Simon K. Davy*

School of Biological Sciences, Victoria University of Wellington, Wellington 6140, New Zealand

*Author for correspondence (simon.davy@vuw.ac.nz)

SUMMARY

The temperature-induced collapse ('bleaching') of the coral–dinoflagellate symbiosis is hypothesised to result from symbiont oxidative stress and a subsequent host innate immune-like response. This includes the production of nitric oxide (NO), which is involved in numerous microbial symbioses. Much of NO's cytotoxicity has been attributed to its conversion, in the presence of superoxide (O_2^-), to highly reactive peroxynitrite (ONOO⁻). However, ONOO⁻ generation has yet to be observed in either a lower invertebrate or an intracellular mutualism. Using confocal laser scanning microscopy with the fluorescent ONOO⁻ indicator aminophenyl fluorescein (APF), we observed strong evidence that ONOO⁻ is generated in symbiotic *Aiptasia pulchella* under conditions known to induce thermal bleaching. However, a role for ONOO⁻ in bleaching remains unclear as treatment with a peroxynitrite scavenger had no significant effect on thermal bleaching. Therefore, while ONOO⁻ may have a potential for cytotoxicity, *in vivo* levels of the compound may be insufficient to affect bleaching.

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Key words: reactive nitrogen species, endosymbiosis, oxidative stress, *Symbiodinium*, cnidarian–dinoflagellate symbiosis, innate immunity.

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INTRODUCTION

Microbial symbioses are ubiquitous in the natural world and sustain some of the most diverse ecosystems on Earth. Among the most ecologically important associations are those between reef corals (Cnidaria) and photosynthetic dinoflagellates. These algal symbionts (genus *Symbiodinium*) provide the host with energy in exchange for nutrients that are typically at low concentrations in the surrounding seawater (Davy et al., 2012). The association underpins the existence of coral reefs but is being placed under increasing strain by recent climatic changes, particularly rising sea surface temperatures (Weis, 2008). It has long been known that excessive heating of corals can result in the loss of their symbiotic algae (a process known as 'bleaching'), yet we still know little about the physiological events underpinning this symbiotic collapse (Weis, 2008; Davy et al., 2012).

Coral bleaching has been linked to the overproduction of reactive oxygen species (ROS) (Lesser, 2006). Often associated with photosynthetic dysfunction, ROS have a well-known capacity for cellular damage (Lesser, 2006), but at low concentrations they also act as signalling compounds (Winterbourn, 2008). It has been hypothesised that ROS leakage from photosynthetically compromised symbionts stimulates an innate immune-like pathway resulting in host nitric oxide (NO) synthesis (Perez and Weis, 2006; Weis, 2008), but very few data exist regarding ROS and cnidarian innate immunity. NO is a ubiquitous signalling compound implicated in the regulation of numerous microbial endosymbioses (Wang and Ruby, 2011). When produced alongside ROS (specifically superoxide, O_2^-), however, NO can convert to highly reactive peroxynitrite (ONOO⁻), which is much more toxic and has a capacity to irreversibly damage mitochondria, antioxidant enzymes, DNA

and lipid membranes (Pacher et al., 2007). In fact, the reaction between NO and O_2^- occurs faster than that of O_2^- with superoxide dismutase, thus the formation of ONOO⁻ is regarded as highly probable when NO and O_2^- are generated simultaneously (Pacher et al., 2007). The likelihood of its generation under thermal stress has therefore led to ONOO⁻ being proposed as the effector of NO-mediated cnidarian bleaching (Perez and Weis, 2006; Weis, 2008).

Using the model cnidarian *Aiptasia pulchella* Carlgren 1943, this investigation sought to test the hypothesis that cnidarian bleaching is dependent on NO's conversion to ONOO⁻ during thermal stress. Filling this gap in our knowledge is important if we are to better understand the cellular basis of coral bleaching and the breakdown of intracellular mutualisms in general.

MATERIALS AND METHODS

Symbiotic specimens of *A. pulchella* were maintained in glass bowls under a 12 h:12 h light:dark cycle (80–100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) provided by cool white fluorescent tubing; OSRAM DULUX L, 36 W 4000K, Osram GmbH, Munich, Germany) at a temperature of 26°C, and fed twice weekly with freshly hatched *Artemia* sp. nauplii. Prior to treatment, anemones were starved for 48 h.

Experimental induction of ONOO⁻ in *Aiptasia pulchella* and its fluorometric assessment

Peroxyntirite was detected using the fluorescent indicator aminophenyl fluorescein (APF; Molecular Probes, Eugene, OR, USA). APF is specific for highly reactive species and detects, alongside ONOO⁻, hydroxyl (OH⁻) and hypochlorite (OCl⁻) radicals. Using specific scavengers of ONOO⁻ and NO, however, one can determine the extent to which APF fluorescence is ONOO⁻

dependent. The suitability of APF was determined by preparing 100 μl solutions of $10 \mu\text{mol l}^{-1}$ APF in 'anemone relaxing solution' [50% 0.22- μm filtered seawater (FSW), 50% 0.37 mol l^{-1} MgCl_2] with and without 2 mmol l^{-1} urate (Sigma-Aldrich, Auckland, New Zealand), a peroxyntirite scavenger that has been employed successfully in fluorescence-based assays (Tewari et al., 2013). The peroxyntirite donor 3-morpholinonydnimine (SIN-1; Life Technologies, Auckland, New Zealand), which generates ONOO^- through the simultaneous release of NO and O_2^- , was then added ($0\text{--}1 \text{ mmol l}^{-1}$ final SIN-1 concentration). APF fluorescence (excitation: 490 nm, emission: 515 nm) was monitored over 2 h using a fluorescent microplate reader (Enspire 2300, Perkin-Elmer, Waltham, MA, USA). To determine whether the effects of urate on APF signal were truly due to scavenging of ONOO^- (rather than quenching of APF fluorescence), the SIN-1/APF incubation was repeated with 2 mmol l^{-1} urate added 30 min after SIN-1.

The ability of APF to detect peroxyntirite *in vivo* was assessed by incubating individual anemones ($N=6$) for 60 min with $10 \mu\text{mol l}^{-1}$ APF in: (1) relaxing solution only, (2) 1 mmol l^{-1} SIN-1, (3) 1 mmol l^{-1} SIN-1 + 2 mmol l^{-1} urate and (4) 1 mmol l^{-1} SIN-1 + 1 mmol l^{-1} of the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (cPTIO; Life Technologies, Auckland, New Zealand).

Endogenous production of ONOO^- by *A. pulchella* was investigated using the high temperature shock (HTS) method. Briefly, anemones (2–3 mm oral disc diameter) in FSW ($N=6$ per treatment in individual glass beakers) were transferred to a 26°C water bath under 12 h:12 h light:dark cycle, (light:

$70\text{--}90 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by an LED light bank; 20 GU10 5 W 6400K, Hispec, Chorley, Lancs, UK) and allowed to acclimate for 48 h [fluorometric assessments of symbiont photosynthesis (see below) were conducted to ensure stability prior to treatment]. Temperature was then increased (over <1 h) to 33°C or kept constant at 26°C (control) and anemones were exposed to these conditions for 24 h. Some additional anemones ($N=6$ per treatment) were HTS-treated in the presence of 2 mmol l^{-1} urate or 1 mmol l^{-1} cPTIO.

To quantify ONOO^- in live *A. pulchella*, the FSW of experimental anemones was replaced with $10 \mu\text{mol l}^{-1}$ APF in relaxing solution and anemones were incubated in the dark for 60 min. Individual anemones were transferred to glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and immobilised with 1% (w/v) low-melting agarose in relaxing solution that had been boiled and cooled to *ca.* 28°C . They were then visualised using an Olympus Fluoview FV-1000 inverted confocal laser scanning microscope (LSM) (Olympus, Center Valley, PA, USA) and $\times 40$ 0.9NA water immersion lens. A 473 nm laser was used to excite APF, the fluorescence of which was measured at 510–530 nm. Symbiont chlorophyll autofluorescence was detected using 635 nm excitation and a 655–755 nm emission filter. Fifteen images in the z -plane were acquired for three tentacles of each replicate anemone and ONOO^- -dependent fluorescence was quantified by measuring the mean 510–530 nm fluorescence intensity of tentacle gastrodermis using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Unstained anemones were processed to control for

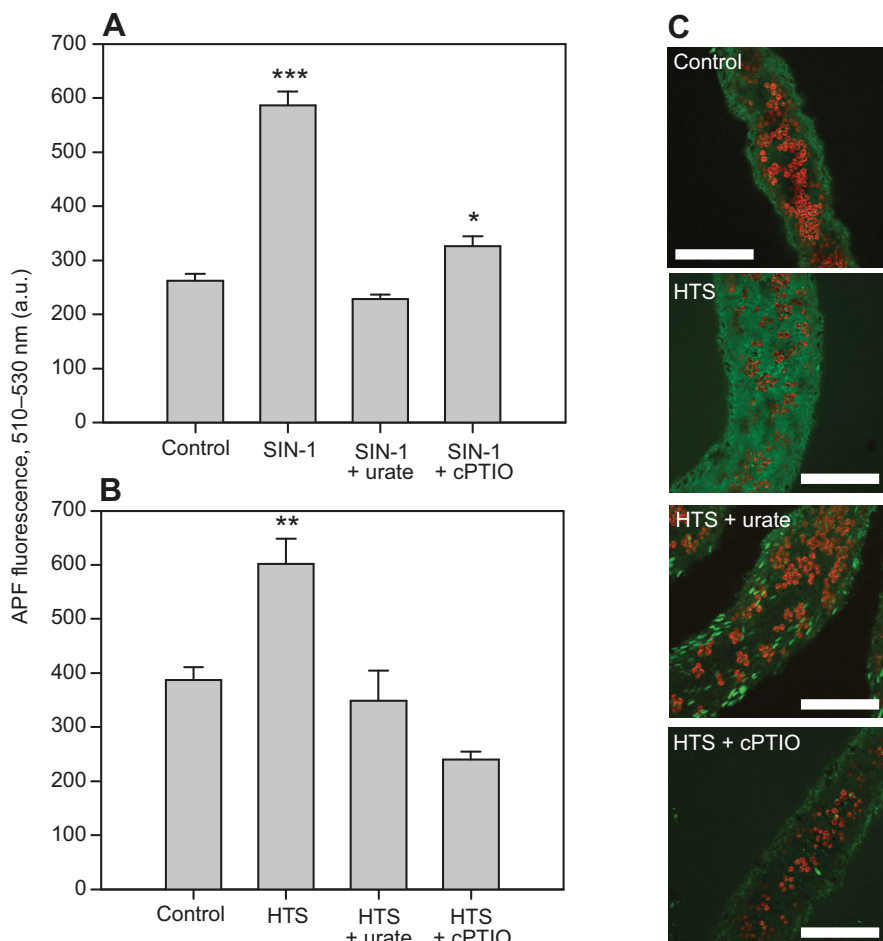


Fig. 1. Detection of peroxyntirite (ONOO^-) in live *Aiptasia pulchella*. Fluorescence intensities of anemone gastrodermis loaded with $10 \mu\text{mol l}^{-1}$ aminophenyl fluorescein (APF) and (A) incubated with and without 1 mmol l^{-1} of the ONOO^- donor SIN-1, 2 mmol l^{-1} of the ONOO^- scavenger urate or 1 mmol l^{-1} of the NO scavenger cPTIO, or (B) exposed to high temperature stress (HTS) with and without 2 mmol l^{-1} urate or 1 mmol l^{-1} cPTIO. Values are means \pm s.e.m. ($N=6$) and asterisks denote significant differences relative to the control group (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). (C) Confocal laser scanning micrographs of anemone tentacles exposed to control conditions, HTS, HTS + 2 mmol l^{-1} urate, and HTS + 1 mmol l^{-1} cPTIO. Green indicates APF fluorescence; symbiont chlorophyll autofluorescence is labelled red. Scale bars, 100 μm .

tissue autofluorescence, and laser intensity/LSM image acquisition settings remained unchanged throughout. Any non-fluorescing anemones were incubated for 30 min with 1 mmol l^{-1} SIN-1 to confirm successful loading of APF.

Examining the role of ONOO⁻ in the thermal bleaching of *Aiptasia pulchella*

The role of ONOO⁻ in temperature-induced bleaching was investigated by incubating *A. pulchella* ($N=5$ in individual glass beakers) for 24 h at 26°C in FSW containing 1 mmol l^{-1} SIN-1 with and without 2 mmol l^{-1} urate. In addition, six replicate anemones were HTS-treated with and without 2 mmol l^{-1} urate. After 24 h exposure to high temperature or SIN-1/urate, anemones were returned to control conditions for a further 24 h (to allow time for bleaching) and then processed for symbiont density assays as described below.

Assessments of symbiont photosystem II fluorescence and host bleaching

Fluorescence yields of photosystem II (PSII) were monitored regularly (midday and 30 min after lights-off) using pulse amplitude modulation fluorometry (Diving-PAM, Walz, Effeltrich, Germany). Symbiont densities were determined as follows. Whole anemones were homogenised using a tissue grinder in a small volume of buffer (50 mmol l^{-1} potassium phosphate, pH 7.8, 1 mmol l^{-1} EDTA). An aliquot was removed for haemocytometer counts (at least six per sample; Improved Neubauer, Boeco, Germany) and the remainder was centrifuged ($16,000g$ for 20 min) and analysed for soluble protein content (Bradford assay). Changes in symbiont density relative to host soluble protein (% symbiont loss) after 48 h were calculated relative to mean pre-treatment ($t=0$) values.

Statistical analysis

Data analysis was carried out using PASW Statistics 18.0 (IBM, Armonk, NY, USA). Where appropriate, data were analysed using repeated-measures ANOVA (RM-ANOVA). RM-ANOVA reports represent time \times treatment interactions. All other analyses were carried out using one-way ANOVA. In all cases data were examined for normality and transformed where necessary.

RESULTS AND DISCUSSION

Detection of ONOO⁻ in symbiotic *Aiptasia pulchella* using APF

APF fluorescence (515 nm) successfully responded to SIN-1-derived peroxynitrite in a dose-dependent manner (supplementary material Fig. S1A). Addition of the ONOO⁻ scavenger urate (2 mmol l^{-1}) prevented this increase, and adding urate after 30 min confirmed that this was due to ONOO⁻ scavenging rather than quenching of fluorescence (supplementary material Fig. S1B). The effects of SIN-1 and urate on APF fluorescence *in vitro* are shown in supplementary material Fig. S1.

Incubation of APF-loaded *A. pulchella* with the peroxynitrite donor SIN-1 resulted in significant increases in tissue 510–530 nm fluorescence (one-way ANOVA, $F_{3,20}=59.753$, $P<0.001$; Fig. 1A) that were absent in the presence of 2 mmol l^{-1} urate or 1 mmol l^{-1} cPTIO (Fig. 1A). HTS treatment of *A. pulchella* also induced increases (one-way ANOVA, $F_{3,19}=14.679$, $P<0.001$), which were absent in the presence of urate or cPTIO (Fig. 1B). The declines in fluorescence intensity when anemones were treated with scavengers either of ONOO⁻ itself (urate) or its precursor NO (cPTIO) confirm that APF signal in *A. pulchella* was an accurate reflection of ONOO⁻

generation rather than the dye's interactions with other highly reactive compounds.

The generation of peroxynitrite (ONOO⁻) has been proposed as a significant step in the cellular cascade underpinning coral bleaching (Perez and Weis, 2006; Weis, 2008) and this study provides strong evidence that ONOO⁻ generation occurs in thermally stressed cnidarians. To the authors' knowledge, it also represents the first observation of ONOO⁻ in either a lower invertebrate (e.g. Porifera, Cnidaria or Ctenophora) or an intracellular mutualism.

Involvement of ONOO⁻ in thermal photoinhibition and bleaching

The addition of urate (2 mmol l^{-1}) significantly alleviated declines in PSII fluorescence yield (RM-ANOVA, $F_{8,60}=12.491$, $P<0.001$; Fig. 2A). Peroxynitrite strongly inhibits mechanisms of electron

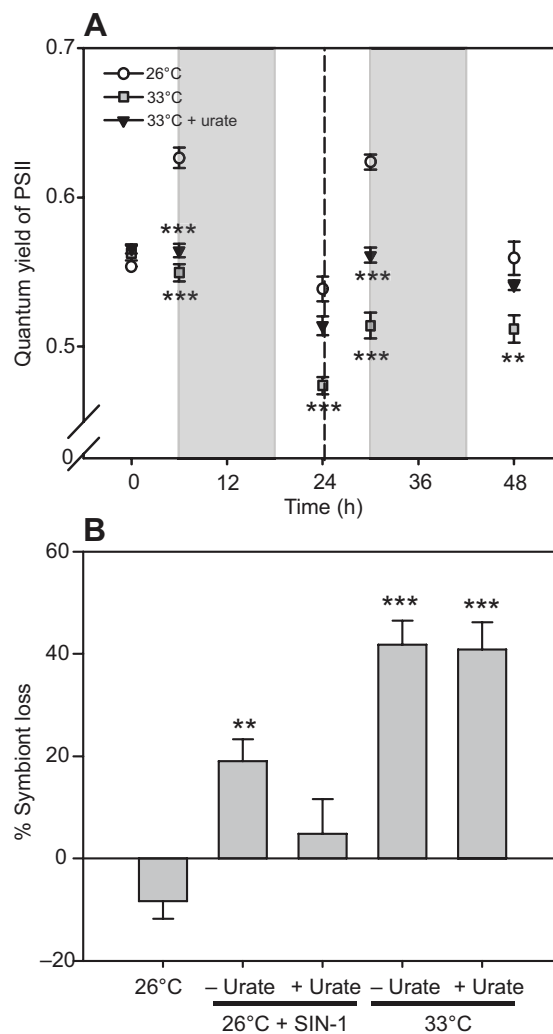


Fig. 2. Involvement of peroxynitrite (ONOO⁻) in temperature-induced photoinhibition and bleaching in *Aiptasia pulchella*. (A) Quantum yields of photosystem II (PSII) over 48 h. Shaded areas represent periods of darkness and the dashed line indicates the time at which anemones were returned to control (26°C) conditions (from 33°C). (B) Percentage bleaching of anemones either treated with 1 mmol l^{-1} of the ONOO⁻ donor SIN-1 or exposed to high temperature stress (HTS; 33°C), both treatments with and without 2 mmol l^{-1} urate. Treatments lasted for 24 h before anemones were returned to control conditions. Values are means \pm s.e.m. ($N=6$ for controls and HTS \pm urate; $N=5$ for SIN-1 \pm urate) and asterisks denote significant differences relative to the control group (** $P<0.01$, *** $P<0.001$).

transport (Pacher et al., 2007) and this presumably includes the components of photosynthesis within symbionts' chloroplasts. As we know very little about the role of reactive nitrogen species in microalgal photoinhibition, this is an attractive area for future work.

ONOO⁻ can be cytotoxic to the host in numerous ways (Pacher et al., 2007) but, in the light of recent investigations (e.g. Dunn et al., 2012), its interactions with cnidarian host mitochondria may be most important. Differences in the reactivity of NO and O₂⁻ and their potential for diffusion mean that ONOO⁻ generation occurs primarily at the sites of O₂⁻ production (Pacher et al., 2007), the most significant of which (in the host at least) is the inner membrane of mitochondria (Lesser, 2006). Current research is linking mitochondrial dysfunction and associated apoptotic pathways to cnidarian bleaching (Dunn et al., 2012), and as noted above, ONOO⁻ is a potent inhibitor of mitochondrial electron transport. However, whether this occurs at *in vivo* ONOO⁻ concentrations has been the subject of debate (Pacher et al., 2007), and we still know little about ONOO⁻ generation beyond mammals. For example, the extremely high reactivity and relatively short half-life of ONOO⁻ in biological systems (Lesser, 2006; Pacher et al., 2007) could very well limit its capacity for diffusion and thus the number of potential target molecules. In this study, the ONOO⁻ donor SIN-1 (1 mmol l⁻¹ in FSW) induced bleaching of *A. pulchella* at control temperatures (one-way ANOVA, $F_{4,27}=20.94$, $P<0.001$; Fig. 2B; *post hoc* Tukey's HSD *versus* control, $P=0.006$) and the ONOO⁻ scavenger urate restored levels of symbiont loss to those of the controls (*post hoc* Tukey's HSD *versus* control, $P=0.365$). It would appear, therefore, that addition of ONOO⁻ to *A. pulchella* can stimulate bleaching. This is not entirely surprising, as a bolus dose of an NO and O₂⁻ donor would be expected to induce significant physiological stress (Lesser, 2006; Weis, 2008). However, there was no significant effect of the ONOO⁻ scavenger urate on thermal bleaching intensity (*post hoc* Tukey's HSD *versus* HTS, $P>0.999$), and this was not due to heat-induced degradation of the scavenger; application to APF/SIN-1 solutions of FSW/urate pre-heated to 33°C for 24 h strongly inhibited APF fluorescence (supplementary material Fig. S1C). Together, these findings suggest that while ONOO⁻ has a bleaching-inducing capacity, either the levels of ONOO⁻ generated *in vivo* are not sufficient to influence symbiont loss, or alternative pathways (perhaps involving ROS or NO directly) are more critical.

In conclusion, we propose that any mediation of temperature-induced cnidarian bleaching by NO occurs independently of its conversion to ONOO⁻. NO has the capacity to directly influence

the cell death pathways implicated in bleaching (Snyder et al., 2009), so ONOO⁻ generation may be unnecessary in this regard. The situation may of course be different in reef corals undergoing bleaching in the field, where light intensities greater than those employed in the present study could exaggerate NO/ROS synthesis in both host and symbiont. In any case, investigating where in the symbiosis ONOO⁻ is produced and how such a potent radical can have such modest effects during temperature stress are important subjects for future study.

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AUTHOR CONTRIBUTIONS

T.D.H. and S.K.D. conceived and designed the experiments. T.D.H. carried out the experiments and subsequent data analysis. T.D.H. wrote the manuscript with guidance from S.K.D.

COMPETING INTERESTS

No competing interests declared.

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