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4C Coral reef response to multiple stresses: organisms to ecosystems

Multiple effects of hydrogen peroxide and temperature on antioxidants and bleaching

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Abstract. This study reports the effect of simultaneous exposure to increased hydrogen peroxide (H_2O_2) levels and high seawater temperature on antioxidant enzyme (SOD and CAT) activities and bleaching in the coral *Galaxea fascicularis*. SOD activities in zooxanthellae were increased by H_2O_2 and a high temperature, but not by H_2O_2 alone. Thus, the increased SOD activity in zooxanthellae after exposure to a high temperature and H_2O_2 levels was caused solely by the high temperature. CAT activities were significantly increased by H_2O_2 and a high temperature in coral tissue and zooxanthellae. However, CAT activities were not increased by higher H_2O_2 levels at a high temperature. Coral and zooxanthellae have a limited capacity to increase their CAT activities. Decrease of zooxanthellae density was observed after exposure to increased H_2O_2 at a high temperature, but zooxanthellae densities were higher than those after exposure to a high temperature alone. Coral bleaching was inhibited by simultaneous exposure to H_2O_2 and a high temperature, despite an oxidative stress level known to damage coral metabolism. The zooxanthellae produced ROS under simultaneous H_2O_2 and high temperature stresses, as suggested by the increased SOD activities. It is likely that the system for trigger of bleaching was unable to act because of the high H_2O_2 concentration in the coral cytosol during H_2O_2 exposure, which led few concentration differences.

Key words: multiple effects, hydrogen peroxide, high temperature, antioxidant enzyme, bleaching

Introduction

Massive coral bleaching caused by environmental changes following both natural events and anthropogenic activities has recently been observed globally. Many studies have reported that elevated seawater temperature is the most important environmental change affecting the coral symbiotic system between the animal hosts and algae, i.e., zooxanthellae (e.g., Brown 1997). Environmental stresses can induce increased production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), leading to significant oxidative damage to the coral-algae symbiotic system (Lesser et al. 1990; Dykens et al. 1992; Downs et al. 2002). Smith et al. (2005) hypothesized that H_2O_2 may be the most important ROS associated with coral bleaching because it can act as an important signaling molecule between *Symbiodinium*, i.e., zooxanthellae, and their symbiotic host.

H_2O_2 is a strong oxidant that is formed photochemically in seawater. The seawater H_2O_2 concentration in the coral reef areas of Okinawa Island can be as high as 0.2-0.5 μM on sunny days (Arakaki et al. 2005, Arakaki et al. 2007). Additionally, because of its high solubility in water, gaseous H_2O_2 in the atmosphere can assimilate into rainwater and fall into the ocean, further increasing H_2O_2 concentrations in seawater (e.g., Zika et al. 1982). Furthermore, the runoff from various human activities into coastal areas also creates substantial stress on coral reefs by degrading seawater quality (Fabricius 2005). Photochemical studies have indicated that H_2O_2 photo-formation was faster in red-soil-polluted seawater than in clean, unpolluted seawater (Nakajima et al. 2004). Nakajima et al. (2004) also reported that H_2O_2 photo-formation rates increased with UV radiation intensity, suggesting that H_2O_2 concentrations in seawater will probably increase in the future due to the thinning of the stratospheric ozone layer that blocks UV radiation. Because of its strong oxidising power, H_2O_2 adversely affects marine organisms. Elevated concentrations of H_2O_2 in the surrounding seawater have been shown to decrease photosynthesis and calcification in the hermatypic coral *Goniastrea aspera* (Higuchi et al., 2009c). Moreover, Higuchi et al. (2009a) reported that the synergistic effect of high temperature and H_2O_2 stresses markedly decreased photosynthesis and calcification, and increased the respiration rate of the coral *Galaxea fascicularis*, compared with each stress alone. However, H_2O_2 in the surrounding water did not induce coral bleaching, even at high concentrations (Higuchi et al. 2009b, 2009c).

The cellular response to the formation of oxygen radicals includes many defense mechanisms (Shick et al. 1995). Enzymes such as superoxide dismutase (SOD) and catalase (CAT) act in concert to inactivate superoxide radicals ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2). A high seawater temperature increased the SOD and CAT activities in host tissue and zooxanthellae (Yakovleva et al. 2004). These enzymes are responsible for detoxifying ROS, and their elevated activities indirectly indicate increased production of ROS in corals as a result of environmental stresses such as temperature, irradiation, and UV radiation (Lesser et al. 1990). Higuchi et al. (2009b) reported that elevated H_2O_2 levels dramatically increased CAT activity in both host and zooxanthellae.

Although the effect of either H_2O_2 or high temperature stresses, alone, has been reported, there is no report of the effect of simultaneous H_2O_2 and high temperature stresses on antioxidant enzyme activities and coral bleaching. Here, the multiple effects of H_2O_2 and high temperature on antioxidant enzyme (SOD and CAT) activities and zooxanthellae density were investigated.

Materials and Methods

Experimental design

Colonies of *Galaxea fascicularis* were collected from a coastal region of Okinawa Island, Japan, with permission from the Okinawa prefectural government (No. 18-11). Corallite was cut and attached to a polycarbonate resin bolt. This experiment was conducted using the same colonies and timing as Higuchi et al. (2009b)

Coral colonies were tested using a continuous-flow complete-mixing (CFCM) experimental system (as described by Fujimura et al. 2008), consisting of a water tank incubation system and a flow-through system, which can continuously supply seawater while maintaining the volume in the incubation tank. Various H_2O_2 concentrations were introduced into the coral incubation tank. The H_2O_2 stock solution was prepared using 0.7 M NaCl + 2 mM $NaHCO_3$ aqueous solution to minimize H_2O_2 decomposition and changes in the salinity and carbonate concentration of the supplied seawater. The H_2O_2 concentration in the stock solution was determined by measuring UV absorbance at 240 nm (ε_{240} = 38.1 M^{-1} cm^{-1}; Miller and Kester 1988). Immediately before the experiments, we prepared 6 and 60 µM H_2O_2 solutions; these were diluted 20-fold in the incubation tank to 0.3 and 3.0 µM, respectively.

Seawater temperature was maintained at 31°C, and two H_2O_2 concentrations (0.3 and 3.0 µM) were applied, each for a five-day period. Six corallites were used per treatment. Light was provided by a metal halide lamp, and photon flux density (200 or 0 µmol m^{-2} s^{-1} during a 12:12 h light/dark cycle) was maintained throughout the experiments.

Isolation of zooxanthellae and determination of antioxidant enzyme activities

Tissue homogenates were prepared using the air-jet method to strip the tissue from the coral skeleton into approximately 10 ml of 100 mmol l^{-1} phosphate buffer (pH=7.0). Homogenate volume was measured, and 1 ml of each subsample was used for determination of zooxanthellae density using direct counts on a Neubauer hemocytometer. The homogenate was further centrifuged twice at 1500 × *g* for 15 min to separate the supernatant and pellets. The supernatant was used for analysis of host coral protein and enzyme activities. The pellets (containing the zooxanthellae) were suspended in 2 ml of 100 mmol l^{-1} phosphate buffer and dissolved by sonication for 30 min in an ice bath. A 0.05% Triton X-100 solution was added to the sonicated suspension. After incubation for 10 min, the suspension was centrifuged at 14000 × *g* for 30 min and used as the algal solution for protein and enzyme assays.

SOD activity was assayed spectro-photometrically as described by Elstner and Heupel (1976) and Oyanagui (1984). Standards were prepared using bovine erythrocytic SOD (Sigma). CAT activity was measured by H_2O_2 depletion at 240 nm (Beers and Sizer 1952). All assays were conducted at 25°C, and enzyme activities are expressed as units (U) per mg protein. Protein content was determined by the Bradford assay (Bradford 1976). Our results were compared with those of Higuchi et al. (2009b), who reported the effect of H_2O_2 or high temperature alone on antioxidant enzyme activities and zooxanthellae density. To investigate the effect of simultaneous H_2O_2 and high temperature stress, a Tukey-Kramer honestly significant difference (HSD) test was used (JMP 8.0, SAS).

Results

Multiple effects on SOD activity

Fig. 1 shows the effect of simultaneous H_2O_2 and high temperature stresses on the SOD activity in coral tissue (a) and isolated zooxanthellae (b). In host tissue, SOD activity was not increased by 0.3 and 3 µM H_2O_2 at a high temperature. In zooxanthellae, SOD activity was increased by approximately 2.5-fold by high temperature stress. SOD was significantly activated by high temperature and both 0.3 and 3 µM H_2O_2 (P <0.05). However, SOD activity was not affected by H_2O_2. There was no significant difference between high temperature stress and simultaneous high temperature and H_2O_2 stresses. Simultaneous

H_2O_2 and high temperature stresses had no significant effect on SOD activities in host coral or zooxanthellae.

Multiple effects on CAT activity

Fig. 2 shows the effect of simultaneous H_2O_2 and high temperature stresses on CAT activity in coral tissue (a) and isolated zooxanthellae (b). CAT activity was significantly increased by high temperature with 0.3 and 3 µM H_2O_2 in coral tissue (P <0.05) compared with the control (27°C, 0 µM). At 31°C with 0.3 µM H_2O_2, the increase in CAT activity in host tissue was reduced compared with other incubation conditions. CAT activity with 0.3 µM H_2O_2 at a high temperature was significantly lower than under other conditions (P <0.05). In zooxanthellae, CAT activity was also significantly increased by high temperature together with 0.3 and 3 µM H_2O_2 (P <0.05), compared with the control. However, H_2O_2 and high temperature showed no synergistic effect on CAT activities in zooxanthellae.

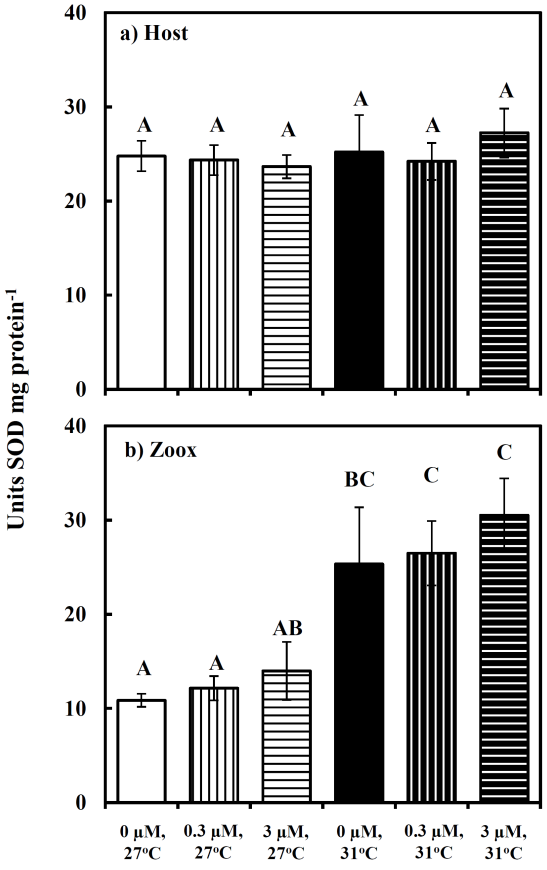

Figure 1: Multiple effects of hydrogen peroxide and temperature on the activity of superoxide dismutase (SOD) in (a) host tissue and (b) zooxanthellae. Means±SE (n=6). The effects of H_2O_2 or high temperature alone (27°C 0, 0.3, 3 µM and 31°C 0 µM) were referred from Higuchi et al. (2009b). Treatments not connected by same letter on the bars are significantly different by Tukey-Kramer HSD test.

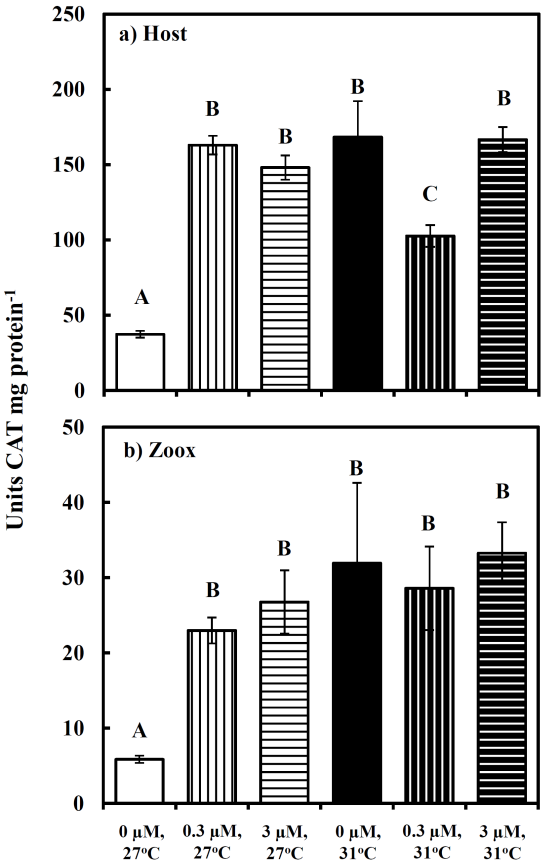

Figure 2: Multiple effects of hydrogen peroxide and temperature on the activity of catalase (CAT) in (a) host tissue and (b) zooxanthellae. Means ± SE (n=6). The effects of H_2O_2 or high temperature alone (27°C 0, 0.3, 3 µM and 31°C 0 µM) were referred from Higuchi et al. (2009b). Treatments not connected by same letter on the bars are significantly different by Tukey-Kramer HSD test.

Multiple effects on coral bleaching

Fig. 3 shows the effect of simultaneous H_2O_2 and high temperature stresses on zooxanthellae density. At a high temperature, zooxanthellae density was decreased by 20 and 29% by 0.3 and 3 µM H_2O_2, respectively, compared with the control (27°C, 0 µM). The density of zooxanthellae exposed to simultaneous H_2O_2 and high temperature stresses was greater than after exposure to high temperature only. There was no significant difference in zooxanthellae density compared with either the control or H_2O_2 stress alone.

Discussion

SOD activities were not increased by H_2O_2 in host coral and zooxanthellae at a high temperature. This was expected since SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2. Thus, the higher

SOD activities in zooxanthellae exposed to a high temperature and H_2O_2 were affected only by the high temperature. Increases in antioxidant enzyme activities are indicative of increased ROS concentrations (Lesser et al. 1990). Yakovleva et al. (2004) showed that the SOD activity of zooxanthellae in *Stylophora pistillata* and *Platygyra ryukyunensis* was increased at high seawater temperatures. Changes in SOD activity respond to alterations in $\bullet O_2^-$ concentration; therefore, the high temperature induced $\bullet O_2^-$ formation in zooxanthellae. In contrast, O_2^- levels in host tissue were not increased by high temperature stress because there was no change in SOD activities. $\bullet O_2^-$ is produced in the mitochondria of host coral at increased temperatures (Downs et al. 2002). SOD activity of *S. pistillata* coral tissue increases at high temperatures, while that of *P. ryukyunensis* coral tissue showed little change (Yakovleva et al. 2004). Thus, the response of SOD activity in host tissue is unclear (i.e., it varies among species). Therefore, the total $\bullet O_2^-$ production in host tissue might be relatively small compared with that in zooxanthellae.

CAT breaks down H_2O_2 into water and oxygen. Increases in CAT activities were the result of increased H_2O_2 concentrations in the host coral and zooxanthellae, which suggest that H_2O_2 in the surrounding seawater reached the cytosol of the coral, as reported by Higuchi et al. (2009b). Dykens and Shick (1982) reported that enzymatic defenses in the animal host are activated in proportion to the potential for photooxidative damage in symbiotic cnidarians. However, despite exposure to multiple stresses, which causes decreased photosynthesis and calcification, and particularly 3 µM H_2O_2 together with a high temperature (Higuchi et al. 2009a), CAT activities were not significantly increased. This is probably because host coral and zooxanthellae have a limited capacity to increase their CAT activities. In contrast, 0.3 µM H_2O_2 together with a high temperature did not increase CAT activity, compared with either stress condition alone, although there was a significant difference compared with the control (27°C, 0µM). Downs and Downs (2007) suggested that the decrease in CAT levels is due to the extreme susceptibility of CAT to oxidation and inhibition by ROS. However, the oxidative stress level used in this study may not have been sufficient to compromise the antioxidant capacity, because marked CAT activity was detected even under the most oxidative condition (3 µM H_2O_2 with a high temperature). Higuchi et al. (2009a) reported that the photosynthesis rate after exposure to 0.3 µM H_2O_2 and a high temperature was higher than that after exposure to a high temperature alone. Nyström et al. (2001) proposed that the response to multiple stressor exposure is combination-specific, where some have antagonistic effects and others additive or synergistic effects. For example, elevated temperature and salinity have mitigating effects on coral metabolism (Porter et al. 1999). Therefore, application of 0.3 µM H_2O_2 might mitigate the effect of high temperature stress, although the mechanism underlying such a mitigation is unknown.

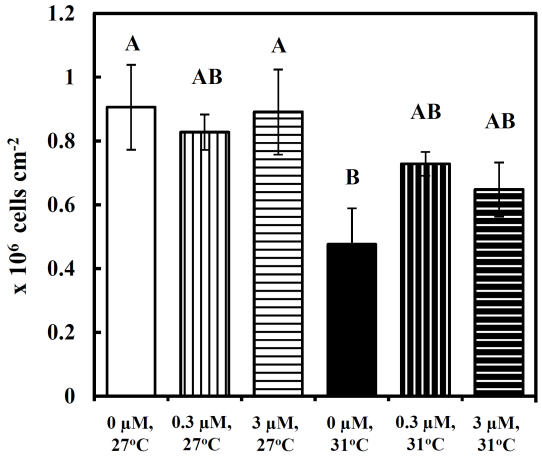

Figure 3: Multiple effects of hydrogen peroxide and temperature on the density of zooxanthellae. Means±SE (n=6). The effects of H_2O_2 or high temperature alone (27°C 0, 0.3, 3 µM and 31°C 0 µM) were referred from Higuchi et al. (2009b). Treatments not connected by same letter on the bars are significantly different by Tukey-Kramer HSD test.

Downs et al. (2002) suggested that bleaching is the final defense of corals against oxidative stress. Ślesak et al. (2007) indicated that H_2O_2 produced in plant cells acts as a signaling molecule in response to environmental stress. However, as shown in Higuchi et al. (2009b), corals (both host coral and zooxanthellae) under increased H_2O_2 oxidative stress were not bleached. These data suggested that H_2O_2 itself cannot directly act as a signaling molecule for bleaching. In contrast, Lesser (1997) reported that simultaneous exposure of corals to the exogenous antioxidants ascorbate and CAT, which scavenge ROS, especially H_2O_2, during temperature-induced stress, prevented bleaching. Thus, H_2O_2 is in some way associated with bleaching. Consequently, coral bleaching is likely to be induced by the H_2O_2 produced by zooxanthellae under stress conditions, but not other H_2O_2 sources (e.g., in the surrounding seawater). Once zooxanthellae produce excess H_2O_2, it is considerable that there is concentration difference between tissue surrounding zooxanthellae and others. Host coral may have a system for trigger of bleaching (e.g. using a concentration difference). Decrease of zooxanthellae density was observed after exposure to increased H_2O_2 at a high temperature (although the difference was not significant), but zooxanthellae densities were higher than those after exposure to a

high temperature alone. Although 0.3 µM H_2O_2 might mitigate the high temperature stress, the zooxanthellae produced ROS under simultaneous H_2O_2 and high temperature stresses, as suggested by the increased SOD activities. Inside the coral cytosol, ROS is inactivated by antioxidant enzymes, such as SOD and CAT. If the antioxidant activity is limited, the final defense mechanism (i.e., bleaching) will be activated. However, coral bleaching was inhibited by simultaneous H_2O_2 and high temperature stresses, despite the presence of an oxidative stress level known to damage coral metabolism, as shown by Higuchi et al. (2009a). It is likely that bleaching system was unable to act because of the high H_2O_2 concentration in the coral cytosol during H_2O_2 exposure, which led few concentration differences.

We report here the effect of simultaneous exposure to increased H_2O_2 and high seawater temperature stresses on antioxidant enzyme (SOD and CAT) activities and bleaching in the coral G. *fascicularis*. Data suggested that the host coral and zooxanthellae cannot further increase their CAT activity even upon exposure to higher oxidative stress levels. Our data confirm that bleaching trigger under high temperature cannot act during H_2O_2 exposure. The multiple effects were unpredictable and so further long-term studies using low-level stresses are required.

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