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Fish & Shellfish Immunology 23 (2007) 188–196

Fish & Shellfish Immunology

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Oxidative burst in hard clam (*Mercenaria mercenaria*) haemocytes

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Received 21 July 2006; revised 11 October 2006; accepted 18 October 2006
Available online 24 October 2006

Abstract

Haemocytes of bivalve molluscs are known to be responsible for many immunological functions, including recognition, phagocytosis, and killing or elimination of invading microorgansisms, such as potentially infective bacteria and parasites. In many bivalves, killing of microorganisms engulfed by haemocytes is accomplished by a sudden release of reactive oxygen species (ROS) within the haemocytes; this response is referred to as an oxidative burst. Previous studies have failed to detect oxidative burst in haemocytes of the hard clam (northern quahog), *Mercenaria mercenaria*. In the present study, we applied a widely used chemical probe for ROS detection in haemocytes, dichlorofluorescin-diacetate (DCFH-DA), to haemocytes from this clam species and used flow cytometry to quantify fluorescence in individual haemocytes. Oxidation of DCFH-DA to the fluorescent product, DCF, within unstimulated haemocytes indicated that ROS were clearly produced in these cells. Two activators of oxidative burst, zymosan and bacterial extracellular products, which have been applied successfully to haemocytes in other species, stimulated large increases in ROS production in hard clam haemocytes. Furthermore, two inhibitors of ROS production, W-13 and diphenylene iodinium (DPI), significantly suppressed ROS production by haemocytes. Nitric oxide synthase inhibitors, NMMA and L-NIO, did not suppress ROS production, indicating that the observed oxidation of DCFH-DA is not mediated by nitric oxide. These results show unequivocally that haemocyte oxidative burst is active in *M. mercenaria* and, therefore, is a likely mechanism in host response to pathogens and parasites.

Keywords: Quahog; Flow cytometry; Haemocyte; Reactive oxygen species; ROS

1. Introduction

In many bivalve species, phagocytic cells can be activated by foreign particles, or organisms and their products, to release oxidative chemicals; this response is often referred to as an ''oxidative burst.'' An oxidative burst leads to production of reactive oxygen species (ROS), catalyzed by the membrane-associated enzyme NADPH oxidase. The initial metabolite, superoxide anion ($O_2^{\cdot -}$) is dismutated to hydrogen peroxide (H_2O_2), which may then be converted

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doi:10.1016/j.fsi.2006.10.006

to other toxic ROS, such as hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$). Hydrogen peroxide also interacts with myeloperoxidase (MPO) and halide to produce hypochlorous acid (HOCl). All of these metabolites likely play an important role in phagocyte-mediated killing of microorganisms [1–3]. ROS act as killing agents, either alone or in combination with lysosomal enzymes, and are important in the elimination of viruses, bacteria, yeast, fungi, and protozoa [4]. Production of ROS in a number of molluscan species has been documented. These species include *Crassostrea virginica*, *Crassostrea gigas*, *Ostrea edulis*, *Mytilus edulis*, *Mytilus galloprovincialis* and *Pecten maximus* [5–8]. The above mentioned studies used luminol-dependent chemiluminescence (CL) measured in a liquid scintillation counter or the optical density of the reduction of nitroblue tetrazolium (NBT) to measure production of ROS. More recent studies have used flow cytometry to measure oxidative burst in *C. virginica* and *C. gigas* [9–12].

Apparent absence of oxidative burst response has been reported in several molluscan species, including *Mercenaria mercenaria*, *Ruditapes decussatus*, *Cerastoderma edule* and *Corbicula japonica* [13–17]. In an early study using measurements of oxygen consumption, biochemical assays, NBT reduction, and detection of MPO, Cheng [13] detected no production of an oxidative burst in actively phagocytic *M. mercenaria* haemocytes. In a more recent study, Anderson [14] used measurements of intracellular $O_2^{\cdot-}$ production by means of NBT reduction and MPO/H_2O_2/halide activity by means of luminol-dependent CL, to confirm an apparent lack of oxidative burst in *M. mercenaria* haemocytes. Anderson et al. [15] later examined haemocyte responses to ROS activators with lucigenin-dependent CL, which measures $O_2^{\cdot-}$ production. The authors concluded that haemocytes of *M. mercenaria* produce basal (resting) ROS activity, but have a very limited capacity to produce an oxidative burst in response to stimuli.

Our investigations of oxidative burst in the hard clam used 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), a fluorescent indicator of oxidative burst measured as H_2O_2 production. When DCFH-DA diffuses into cells, it is hydrolyzed by intracellular esterases to form the non-fluorescent derivative, 2′,7′-dichlorofluorescin (DCFH), which is retained within the cell membrane. In the presence of hydrogen peroxide, this compound is oxidized to the highly fluorescent, intracellular product 2′,7′-dichlorofluorescein (DCF). Intracellular oxidation of DCFH-DA is quantitatively related to oxidative burst in haemocytes [10,18–20]. The production of ROS was investigated using flow cytometry, which can provide information about oxidative burst at the single-cell level. Our results indicated that, by use of this method, *M. mercenaria* haemocytes can be shown to posses oxidative burst capability. This report explores several methods of activating and inhibiting oxidative burst in haemocytes of *M. mercenaria*.

2. Materials and methods

2.1. Haemolymph sampling

Hard clams were collected from local waters by a recreational diver and maintained in the NOAA Milford Laboratory for several weeks in running, unfiltered seawater. In all experiments, haemolymph was withdrawn from the adductor muscle with a 25-gauge needle and 1-ml plastic syringe. Haemolymph from each animal was transferred to an individual Eppendorf tube held on ice to limit spontaneous activation and reduce clumping before use. Haemolymph samples were examined microscopically for contamination with cells from other tissues. In some experiments, haemolymph from three or more clams was pooled; in others, haemolymph from individual clams was analysed separately.

2.2. Flow cytometry

Samples from each assay were analysed in 5-ml flow-cytometer tubes with a BD BioSciences (San Jose, CA) FACScan flow-cytometer. Data were later analysed using the flow cytometry analysis software WinMDI 2.8 or BD Cell Quest, and oxidative burst was calculated as the difference in geometric mean fluorescence of activated and non-activated haemocyte samples.

To test whether *M. mercenaria* haemocytes are capable of being stimulated to produce an oxidative burst, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) was employed as a fluorescent indicator of oxidative burst. The green fluorescence produced by DCF was measured by the FL1 detector of the flow-cytometer.

2.3. Activation with zymosan

To prepare a zymosan A stock solution, 200 mg zymosan A (Sigma Z4250) was suspended in 10 ml filter-sterilized seawater (FSW), the solution was heated in a boiling water bath for 30 min, and washed twice by centrifugation. The stock suspension was re-suspended in a range of volumes of FSW to make three suspensions that contained increasingly higher concentrations of zymosan particles. We used zymosan A suspensions to induce oxidative burst, following the general protocol developed by Lambert et al. [10] for *Crassostrea gigas* and Hégaret et al. [9] for *Crassostrea virginica*.

Aliquots of 400 μl haemolymph from each clam (in triplicate) were diluted approximately ten-fold in marine molluscan saline (MMS) (0.58 M NaCl, 0.013 M KCl, 0.013 $CaCl_2$, 0.026 M $MgCl_2$, 0.00054 M Na_2HPO_4, 0.05 M Tris–HCl buffer, at pH 7.6). Diluted haemolymph (900 μl) was transferred to each of four flow-cytometer tubes, and tubes were incubated with 10 μl DCFH-DA (Calbiochem 287810) (0.1 mM final concentration), and 100 μl of each zymosan A suspension. In controls, zymosan A suspensions were substituted with 100 μl FSW. The final haemocyte-to-zymosan ratios were approximately 1:1, 1:10, and 1:100. All tubes were incubated in the dark at room temperature (20 °C) for 5 min before analysis with the flow cytometer. Activation by zymosan A was measured as the difference in fluorescence between activated and non-activated samples.

In another assay, zymosan A was used to activate the oxidative burst of the haemocytes at a final haemocyte-to-zymosan ratio of approximately 1:100. W-13, a calmodulin inhibitor and an inhibitor of the NADPH-oxidase system, was used as an inhibitor of oxidative burst [12]. W-13 (Sigma A0666) was prepared at 1 mg ml^{-1} in sterile, distilled water. Six clams were analysed individually to explore variability between individual clams. Haemolymph from each clam (100 μl) was incubated with either: (1) 40 μl zymosan A plus 260 μl FSW, to test the effect of an activator, or (2) 15 μl of the inhibitor W-13, plus an additional 285 μl FSW, or finally (3) 300 μl FSW as a control. Four microlitres of DCFH-DA (1 mg ml^{-1}) was added to each of the tubes at the beginning of the experiment. All tubes were incubated in the dark at room temperature (20 °C) for 2 h before analysis with the flow cytometer. Activation with zymosan A and inhibition by W-13 were measured as the difference in cell fluorescence between activated or inhibited samples and non-activated samples.

2.4. Activation with ECP

Extracellular products (ECP) of the bacterium *Vibrio aestuarianus*, strain 01/32, were also used as activator of the oxidative burst response in the clam *M. mercenaria*. ECP were shown to activate the production of ROS in Pacific oyster (*C. gigas*) haemocytes [11,21]. ECP were provided by Yannick Labreuche (IFREMER, Brest, France) and prepared according to Labreuche et al. [11]. Diphenylene iodinium (DPI), an NADPH-oxidase inhibitor, was used to inhibit oxidative burst following the method by Delaporte et al. [22]. Since the NADPH-oxidase system mediates the production of ROS, inhibition of NADPH would suppress production of all ROS [23].

Haemolymph from three clams was pooled to yield each of three replicate pools used for analyses. Haemolymph from each pool (100 μl) was incubated with 300 μl FSW as a control, or with 300 μl FSW containing a final concentration of ECP equivalent to 30 bacteria ml^{-1}. Finally, the inhibition of ROS production by DPI was measured by adding 300 μl FSW containing 5 μM DPI to 100 μl haemolymph. Each tube received 4 μl DCFH-DA (1 mg ml^{-1}) at the beginning of the experiment. All tubes were incubated in the dark at room temperature (20 °C) for 2 h before analysis with the flow cytometer.

In another assay, ROS production was activated with two concentrations of ECP. Haemolymph from three clams was pooled to yield each of three replicate pools used for analyses and distributed into three different treatments. Aliquots of 100 μl haemolymph were added to either: (1) 300 μl FSW, or (2) 300 μl FSW containing a final concentration of ECP equivalent to 30 bacteria ml^{-1} or finally, (3) 300 μl FSW containing a final concentration of ECP equivalent to 60 bacteria ml^{-1}. Four microlitres of DCFH-DA (1 mg ml^{-1}) was added to each of the tubes at the beginning of the incubation. Tubes were maintained in the dark at room temperature (20 °C) for 2 h before analysis with the flow cytometer.

The effect of the duration of incubation with ECP was also measured. Haemolymph was withdrawn from four individual clams and distributed into two different treatments. Aliquots of 100 μl of haemolymph were added to either: (1) 300 μl FSW, or (2) 300 μl FSW containing a final concentration of ECP equivalent to 30 bacteria ml^{-1}. Four

microlitres of DCFH-DA (1 mg ml^{-1}) was added to each of the tubes at the beginning of the incubation. Tubes were maintained in the dark at room temperature (20 °C), for 0, 10, 70 and 120 min before analysis with the flow cytometer.

2.5. Nitric oxide synthase inhibitors NMMA and L-NIO

Nitric oxide (NO) synthase inhibitors N^G-methyl-L-arginine acetate salt (NMMA, Sigma M7033) and L-N^5-(1-iminoethyl)-ornithine−2HCl (L-NIO, Alexis Biochemicals 270-002-M025) were used to determine if NO contributes to the oxidation of DCFH to DCF. NMMA and L-NIO are arginine analogs that inhibit the production of NO [12,19,23]. NMMA and L-NIO were prepared at 1 mg ml^{-1} and 16.7 mg ml^{-1}, respectively, in sterile, distilled water. Haemolymph was withdrawn from six individual clams and distributed into four different treatments. Aliquots of 100 μl haemolymph diluted with 100 μl FSW were added to either: (1) 200 μl FSW, (2) 132 μl FSW plus 68 μl ECP, (3) 122 μl FSW, plus 68 μl ECP and 10 μl NMMA, or finally, (4) 127 μl FSW, plus 68 μl ECP and 5 μl L-NIO. Four microlitres of DCFH-DA (1 mg ml^{-1}) was added to each of the tubes at the beginning of the incubation. Tubes were maintained in the dark at room temperature (20 °C) for 2 h before analysis with the flow cytometer.

2.6. Data analysis

Flow-cytometer haemocyte data were collected for 30 s at the ''high'' flow setting on the instrument, resulting in files containing measurements from 5000–10,000 haemocytes. Percent differences in DCF fluorescence produced by haemocytes in experimental treatments, compared to control haemocytes incubated with FSW, or means of replicate values of DCF fluorescence were compared with one-way ANOVA or MANOVA tests using Statgraphics Plus (Manugistics, Rockville, MD) software. Percent differences in fluorescence were Arcsin of the square root transformed before statistical analysis.

3. Results

ROS production was measured by the oxidation of non-fluorescent DCFH-DA to fluorescent DCF. Results from the first assay indicated that oxidative burst in *M. mercenaria* haemocytes can be activated by zymosan A (Fig. 1) and that a higher concentration of zymosan particles (1:10–1:100) stimulated a stronger oxidative burst. Whereas ROS production could be activated by zymosan A, W-13 effectively inhibited ROS production (Fig. 2a). ECP also activated ROS production; whereas, DPI inhibited ROS production to below basal levels (Fig. 2b).

As the production of ROS was activated when haemocytes were incubated with ECP, we investigated whether or not this activation was dose-dependent. The mean ROS content of haemocytes incubated in FSW was significantly lower than that of haemocytes incubated in ECP, but there was no significant difference between the two ECP treatments (Fig. 3a). Thus, a concentration of ECP equivalent to 30 bacteria ml^{-1} seems to be sufficient; twice this concentration of ECP did not induce higher ROS production.

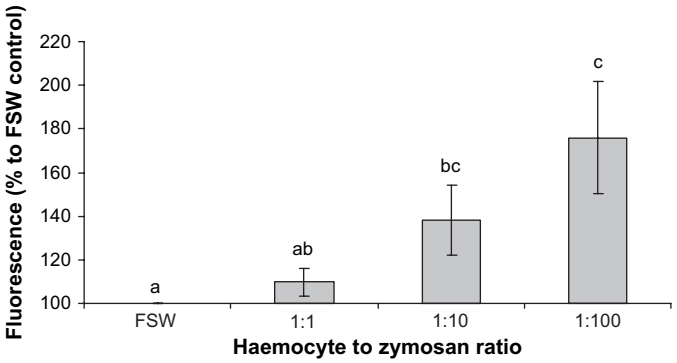

Fig. 1. Percent differences in production of ROS in haemocytes exposed to zymosan A at different haemocyte:zymosan A ratios (1:1, 1:10, 1:100) as compared to haemocytes incubated with FSW (mean ± SEM, $n = 6$ clams). Different lower case letters indicate significant differences between haemocyte treatments ($p < 0.05$, ANOVA).

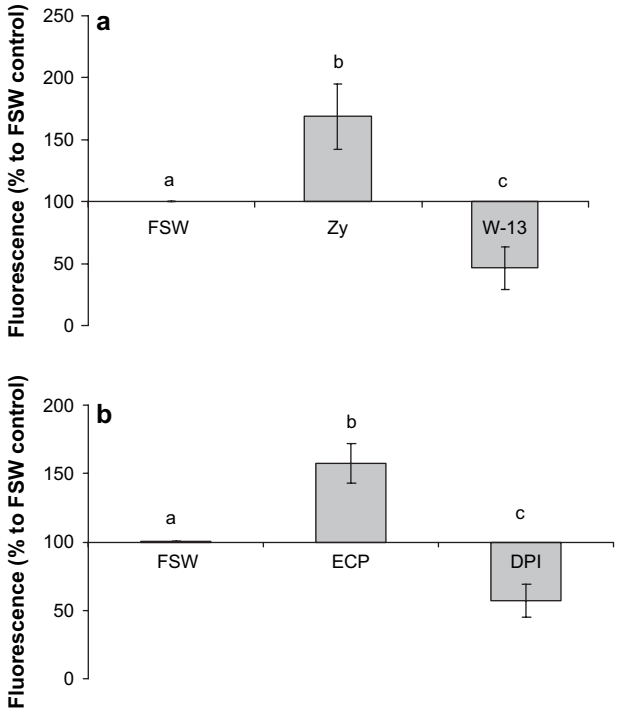

Fig. 2. (a) Percent differences in the production of ROS in haemocytes exposed to either an activator (zymosan A) or an inhibitor of oxidative burst (W-13) compared to haemocytes incubated with FSW (mean ± SEM, $n = 6$ clams). (b) Same as (a), but with ECP as an activator and DPI as an inhibitor (mean ± SEM, $n = 3$ haemolymph pools). For each graph, different lower case letters indicate significant differences between haemocyte treatments (ANOVA, $p < 0.001$).

Other experiments demonstrated that the fluorescent signal of DCF is cumulative until 70 min of incubation. The oxidative burst response was initiated during the first 10 min, and ROS rose to a maximal level after 70 min, with no further increase after 120 min (Fig. 3b). Thus, to measure the production of ROS and activation with ECP, 70 min of incubation is optimal.

Results from the final experiment showed that the nitric oxide synthase inhibitors NMMA and L-NIO did not suppress oxidative burst in *M. mercenaria* haemocytes (Fig. 4).

4. Discussion

Results from our experiments indicate that the oxidative burst defense mechanism is present in the hard clam and can be measured with DCF fluorescence. Haemocytes from *M. mercenaria* produce basal levels of ROS when unstimulated and can be stimulated to produce an oxidative burst. Results show that *M. mercenaria* haemocytes increase their ROS production when stimulated with zymosan A or ECP. The NADPH-oxidase inhibitor DPI appears to inhibit oxidative burst, indicating that the oxidation of DCFH-DA to DCF, thus the oxidative burst response, is dependent upon NADPH oxidase [22–24]. DCFH oxidation is widely used to measure H_2O_2 production, however, there is some evidence that nitric oxide (NO) and peroxynitrite ($ONOO^-$), which is produced by an interaction between NO and O_2^- [25], may also be capable of oxidizing DCFH [26,27]. Although we have not directly demonstrated NO production in *M. mercenaria* haemocytes, nitric oxide synthase inhibitors were used in order to rule out the possibility that NO might be responsible for the measured oxidative bust. Failure of two nitric oxide synthase inhibitors, NMMA and L-NIO, to suppress oxidative burst indicate that oxidation of DCFH-DA is not mediated by nitric oxide [12,21]. Incubation with W-13 resulted in an inhibition of ROS production. As W-13 inhibits $O_2^{\cdot-}$ and subsequent H_2O_2 production, inhibition by W-13 indicates that H_2O_2 is the predominant species involved in the oxidation of DCFH [12].

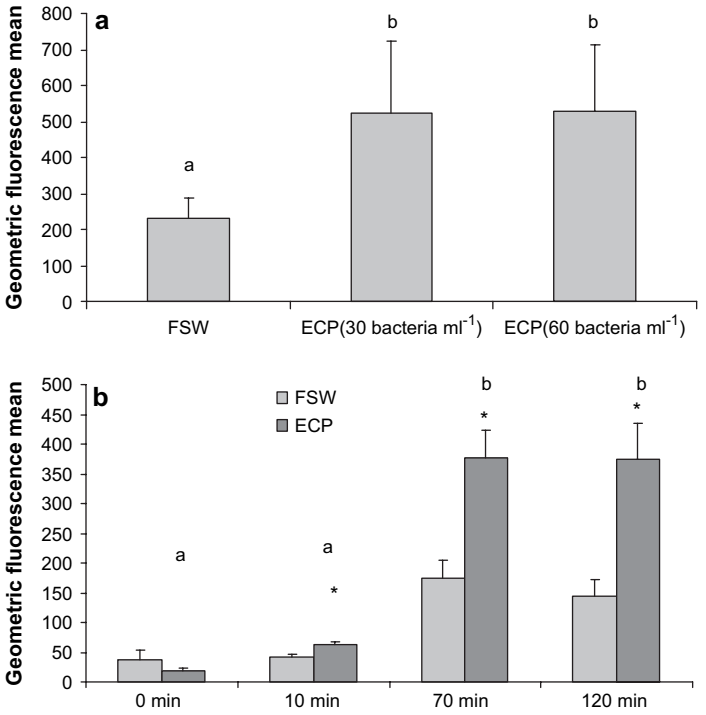

Fig. 3. (a) Production of ROS in haemocytes incubated in FSW, or exposed to bacterial extracellular products (ECP) used here as an activator of oxidative burst at two different concentrations (mean ± SEM, $n = 4$ haemolymph pools). Different lower case letters indicate significant differences between haemocyte treatments (ANOVA, $p < 0.05$). (b) Production of ROS in haemocytes incubated in FSW or exposed to ECP for a range of incubation times (mean ± SEM, $n = 4$ clams). MANOVA analyses showed a significant effect of the activator ($p < 0.001$), a significant effect of incubation time ($p < 0.001$), and a significant interaction between the time of incubation and the activator ($p < 0.001$). Different lower case letters indicate significant differences between incubation times and asterisks indicate significant differences between haemocyte treatments.

The inter-individual variation in DCF fluorescence associated with the production of ROS in clam haemocytes can be quite large; therefore, mean values for several individual clams generally show very high variance. In contrast, comparisons of haemocytes from individual clams treated with activators and inhibitors show very large and obvious effects. Other investigations of oxidative burst in molluscs have encountered difficulties with reproducibility between different haemocyte samples [1]. However, the results of the present study demonstrate clearly that oxidative burst is present in this species, and that the oxidative burst response can be stimulated by more than one activator.

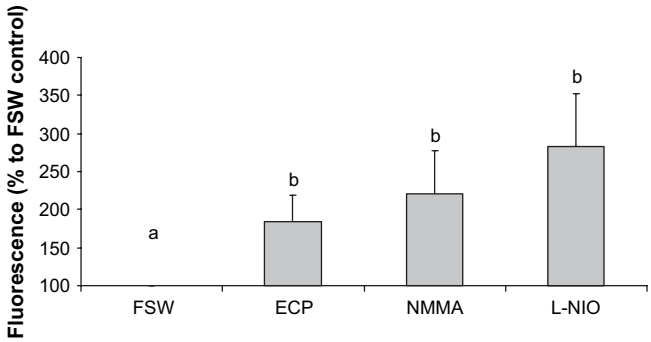

Fig. 4. Percent differences in the production of ROS in haemocytes exposed to an activator of oxidative burst (ECP) or to ECP plus either of the nitric oxide synthase inhibitors NMMA or L-NIO as compared to haemocytes incubated with FSW (mean ± SEM, $n = 6$ clams). Different lower case letters indicate significant differences between haemocyte treatments (ANOVA, $p < 0.001$).

Previous investigations of *M. mercenaria* may have failed to detect an oxidative burst because the methods used to activate ROS production were not effective for this species. In an NBT reduction assay, phorbol myristate acetate (PMA) was used to stimulate ROS production in haemocytes [14]. Our assays, however, indicate that PMA may not be a good activator for *M. mercenaria* haemocytes (data not shown). Authors have shown that PMA activates an oxidative burst in *C. virginica* haemocytes [12], whereas others have indicated that bivalve haemocytes are less sensitive to PMA than to zymosan A [3,10,28]. Cheng [13] attempted to activate haemocytes with heat-killed vegetative cells of *Bacillus megaterium*, and failed to detect an oxidative burst. It is possible that the bacterium is not an effective stimulator of oxidative burst in *M. mercenaria* haemocytes or that the bacterium:haemocyte ratios were too low to allow for activation. In order to produce a detectable oxidative burst, as many haemocytes as possible should come into contact with the stimulus. A low number of stimulatory particles may prevent many haemocytes from being activated, resulting in an undetectable oxidative burst response.

Media composition that is unfavorable to hard clam haemocytes may also have played a role in the failure to detect oxidative burst in previous assays. For example, the phagocytic activity of *Lymnaea stagnalis* haemocytes was found to be very low in phosphate buffered saline (BPS) as compared to that in physiological snail saline [1], and production of ROS in *Mytilus galloprovincialis* was inhibited by modified Alsever's solution (MAS) [26]. Glucose, a component of MAS, was also found to inhibit *M. galloprovincialis* haemocyte ROS production, most likely because of its ability to scavenge radicals [29,30]. Glucose is also a component of Hanks' balanced salt solution, which was used in some of the previous assays attempting to detect oxidative burst in *M. mercenaria* [14]. It is possible that the presence of glucose in the experimental medium further reduced the already low levels of ROS produced by *M. mercenaria*, resulting in an insignificant response to zymosan.

Several different chemical probes were used to detect oxidative burst in previous experiments with *M. mercenaria* haemocytes. Commonly used probes measure different reactive oxygen species: lucigenin-dependent chemiluminescence and NBT reduction are widely used to measure production of $O_2^{\cdot-}$ [1], luminol-dependent chemiluminescence is used to measure the activity of the MPO/H_2O_2/halide system [31]; whereas, DCF fluorescence is used to measure H_2O_2 [18]. The method chosen to detect ROS production must be sensitive enough to compete with antioxidant compounds for reaction with the particular ROS [32], and results may vary among different methods. Others have observed differences in measurements of oxidative activity in haemocytes of the razor clam (*Siliqua patula*) dependent upon the probe used to detect ROS [33].

None of the previous investigations of oxidative burst in *M. mercenaria* used DCF fluorescence to measure ROS production. The reaction of DCFH with H_2O_2 may be more sensitive than reactions of other probes with other ROS; this sensitivity may be responsible for our ability to detect an oxidative burst response. Additionally, the ROS probe must measure intracellular ROS production in order to most accurately measure intracellular antimicrobial potential. The DCF/flow cytometry technique is known to measure intracellular H_2O_2, whereas, a recent study found that lucigenin does not penetrate cells, indicating that lucigenin-augmented CL measures extracellular $O_2^{\cdot-}$ production [34]. In the case of *M. mercenaria*, lucigenin-augmented CL may fail to accurately reflect ROS production within haemocytes; however, it is useful in determining the superoxide generating capacity of the cells. However, NBT reduction assays measure intracellular superoxide, as evidenced by the formation of formazan deposits in the cells. No significant increased NBT reduction could be shown in the case of PMA-activated *M. mercenaria* haemocytes, as compared to *C. virginica* [14].

The use of flow cytometry in our assays permitted the measurement of oxidative burst on an individual-cell level. The greater sensitivity of this technology may have contributed to our ability to detect oxidative burst in *M. mercenaria* haemocytes. Techniques similar to ours may be used to re-evaluate whether oxidative burst is present in species previously described as not possessing this defense capability.

Evidence that *M. mercenaria* haemocytes are capable of producing an oxidative burst in response to stimuli changes our understanding of the defense mechanisms of these animals. The assays we used to demonstrate ROS production in clam haemocytes can be used to develop future experiments to clarify the role of ROS in hard clam, haemocyte-mediated, microbicidal activity and the pathogenesis of hard clam diseases.

Acknowledgements

The authors thank R. Anderson for his review of a prior draft of this manuscript. This research was supported by the New York State Department of Environmental Conservation and is also a resulting product from project R/FBF-17

funded by New York Sea Grant. The statements, findings, conclusions, views, and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations.

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