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NADPH Oxidase-like Activity in Hemocytes of the Pacific Oyster *Crassostrea gigas*

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ABSTRACT — To test whether NADPH oxidase-like activity in hemocytes of the Pacific oyster, *Crassostrea gigas*, occurs, we investigated molecular oxygen (O_2) consumption and superoxide anion (O_2^-) generation during the respiratory burst. When oyster hemocytes were stimulated with phorbol myristate acetate (PMA), a rapid increase in O_2 consumption was recorded accompanying a cyanide-independent respiratory burst using a Clark-type oxygen electrode. The O_2 consumption was almost completely inhibited by the addition of 2 μM diphenyleneiodonium (DPI), a specific inhibitor of mammalian NADPH oxidase. Oyster hemocytes, on stimulation with PMA, exhibited a relatively strong O_2^--dependent chemiluminescent (CL) response at the peak photocount of 4.67×10^5 CPM/2×10^6 hemocytes. The CL response induced by PMA-stimulated hemocytes was markedly reduced by 88% in the presence of 2 μM DPI. Furthermore, O_2^- generation was measured by the reduction of acetylated ferricytochrome *c*. When the primary O_2^- generation by PMA-stimulated hemocytes was terminated by detergent mediated cell lysis, and reconstituted by the addition of exogenous NADPH, O_2^- generation in the hemocyte lysate was restored. Findings obtained in our experiments suggest that an NADPH oxidase-like activity, which is associated with a specific O_2^--forming system similar to that in mammalian phagocytes, exists in oyster hemocytes.

Key words: NADPH oxidase, Pacific oyster, hemocyte, superoxide anion, *Crassostrea gigas*, chemiluminescence, oxygen consumption

Mammalian neutrophils and macrophages markedly increase molecular oxygen (O_2) consumption and generate reactive oxygen intermediates (ROI) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (· OH) when these cells respond to phagocytic particles or soluble stimuli such as zymosan or phorbol myristate acetate (PMA) (reviewed by Babior, 1984). In this respiratory burst process, an initial biochemical event in ROI production is the conversion of O_2 to O_2^- catalyzed by the NADPH oxidase system (Makino *et al.*, 1986).

The NADPH oxidase system in mammalian neutrophils and macrophages is known to be composed of serial redox components, i.e., NADPH, cytochrome b_{558}, a flavoprotein, two cytosolic proteins, $p47^{phox}$ and $p67^{phox}$, and the small GTP-binding protein, $p21^{rac}$ (reviewed by Segal and Abo, 1993). Also in many fish species, ROI are apparently generated by neutrophils and macrophages (reviewed by Secombes and Fletcher, 1992), and an azide-independent respiratory burst was demonstrated for macrophages of rainbow trout, *Oncorhynchus mykiss* (Nagelkerke *et al.*, 1990). The occurrence of NADPH oxidase-like activity in rainbow trout macrophages has also been reported (Secombes *et al.*, 1992). Moreover, Itou *et al.* (1996) demonstrated the kinetics of oxygen metabolism in neutrophils of the Japanese eel, *Anguilla japonica*, was similar to that of mammals. Additionally, the large subunit of cytochrome b_{558} was extensively detected in fish neutrophils (Itou *et al.*, 1998; Shiibashi *et al.*, 1999).

Recent investigations have demonstrated the production of ROI by stimulated hemocytes of several bivalve molluscs: O_2^- and/or H_2O_2 generation by hemocytes from the mussel, *Mytilus edulis* (Pipe, 1992; Noël *et al.*, 1993), the eastern oyster, *Crassostrea virginica* (Larson *et al.*, 1989; Anderson *et al.*, 1992a; Austin and Paynter, 1995); the Pacific oyster, *C. gigas* (Bachère *et al.*, 1991; Takahashi *et al.*, 1993); the European flat oyster, *Ostrea edulis* (Bachère *et al.*, 1991); the scallop, *Patinopecten yessoensis* (Nakamura *et al.*, 1985). The oxidative activity in molluscan hemocytes

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has been shown to be valuable in defining host-defensive events (Adema *et al.*, 1991; Anderson, 1994; Greger *et al.*, 1995) and hemocyte-pathogen interactive modulation (Le Gall *et al.*, 1991; Anderson *et al.*, 1992b; Bramble and Anderson, 1997, 1998).

In the previous paper, we demonstrated using electron spin resonance (ESR) spin trapping and by the chemiluminescence method that Pacific oyster hemocytes stimulated with PMA generated exclusively O_2^- as the primary O_2 metabolite (Takahashi *et al.*, 1993). Chemiluminescent response depending on ROI generation by zymosan-stimulated hemocytes of the pond snail, *Lymnaea stagnalis* and *C. virginica* was strongly inhibited by the addition of diphenyleneiodonium (DPI) (Adema *et al.*, 1993; Bramble and Anderson, 1999). DPI is a potent inhibitor of mammalian NADPH oxidase (Cross and Jones, 1986), and also inhibits the activity of an NADPH oxidase-like enzyme in rainbow trout macrophages (Secombes *et al.*, 1992). Therefore, the ROI production in *L. stagnalis* and *C. virginica* hemocytes probably depend on NADPH oxidase (NADPH oxidase-like enzyme) activity (Adema *et al.*, 1993; Bramble and Anderson, 1999). In *C. gigas* hemocytes, however, some of the details of respiratory burst and NADPH oxidase systems have yet to be unequivocally demonstrated. In the present paper, we aimed to show the occurrence of NADPH oxidase-like activity in Pacific oyster hemocytes.

Materials and Methods

Chemicals

2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-a]pyradin-3-one hydrochloride (MCLA), which has a higher specificity than the conventionally used luminol for detection of O_2^-, was purchased from Tokyo Kasei Kogyo, Japan. DPI chloride was obtained from Dojindo Laboratories, Japan. DPI was dissolved in dimethylsulfoxide (DMSO, Nacalai Tesque, Japan) at a concentration of 1.6 mM; working solution was diluted in a balanced salt solution for oyster hemocytes (oyster BSS: NaCl, 446.6 mM; KCl, 14.5 mM; $CaCl_2$, 8.6 mM; $MgCl_2$, 10.6 mM; $MgSO_4$, 14.2 mM; $NaHCO_3$, 3.0 mM; NaH_2PO_4, 0.08 mM; glucose, 5.6 mM; pH 7.8). PMA, Cu, Zn-superoxide dismutase (SOD, derived from bovine erythrocytes), ferricytochrome *c* (type VI), NADPH (tetrasodium salt, type X), and deoxycholate (DOC) were purchased from Sigma Chemical Co., USA.

Oysters

Two-year-old Pacific oyster, *C. gigas*, specimens ranging from 11 to 15 cm in shell height, were harvested from hanging cultures in Matsushima Bay, Miyagi Prefecture. They were held in 150 L aquaria with recirculated, filtered artificial seawater (MARINE ART BR, Senju Seiyaku, Japan) for 2–5 days. Water temperature was maintained at 15 ± 1°C.

Isolation of oyster hemocytes

Hemolymph was withdrawn from the blood sinus in the adductor muscle using a tuberculin syringe with 23 gauge, 1.5 inch needle. Each oyster was bled once only. Hemolymph was centrifuged at 290 × *g* for 20 min at 4°C to separate the hemocytes. The resulting hemocyte pellet was washed three times with ice-cold oyster BSS and the final hemocyte pellet resuspended to 5×10^7 cells/mL in oyster BSS. To minimize cell clumping, the hemocyte suspension was maintained on ice until use.

Effect of DPI on the PMA-stimulated O_2 consumption of oyster hemocytes

O_2 consumption was measured by using a membrane-covered Clark-type oxygen electrode (Model 5300, YSI Inc., USA), under constant agitation with a magnetic stirrer. Oyster hemocytes (1×10^7 cells) were suspended in 200 μL of modified Krebs-Ringer solution containing 446.6 mM NaCl and 5.6 mM glucose (KRG, pH 7.4). To the hemocyte suspensions 100 μL of 2 mM KCN was added in order to inhibit the respiratory activity of mitochondria. The reaction was started by the addition of 100 ng/mL of PMA (100 μL). DPI (2 μM, final concentration) was added at 3.5 min after the addition of PMA to the hemocyte suspension. All measurements were carried out at 23°C.

Influence of DPI on MCLA-enhanced chemiluminescence

Chemiluminescence (CL) procedure was almost the same as described by Nishida *et al.* (1989). Reaction mixtures contained 2×10^6 oyster hemocytes, 3 μM MCLA , 0 or 2 μM DPI and oyster BSS in a total volume of 2.0 mL. A Luminescence Reader (BLR-301, Aloka, Japan) was used to detect MCLA-enhanced CL activity. The reaction was initiated by the addition of 100 ng/mL of PMA. During the CL measurement, the reaction mixtures were agitated by rotation at 26°C in the Luminescence Reader.

Detection of O_2^- generation by the reduction of acetylated ferricytochrome c

Acetylated ferricytochrome *c* was prepared by the procedure of Tsunawaki and Nathan (1984). The efficiency of the acetylated ferricytochrome *c* in detecting O_2^- released from PMA-stimulated hemocytes was 45.5% of that of native cytochrome *c*. Oyster hemocytes (1×10^7 cells) were suspended in 1.0 mL of KRG with acetylated cytochrome *c* and 2 mM NaN_3, an inhibitor of cytochrome oxidase, in a 1-cm light path cuvette. The increase in absorbance at 550–540 nm ($\Delta A_{550-540}$) was continuously recorded with a spectropho-

tometer (Hitachi U-2000, Japan) at 23°C. The optical density was converted to nanomoles of cytochrome *c* reduced by using the molar absorption coefficient of 19.1 mM^{-1} cm^{-1} (Kakinuma and Minakami, 1978). The reaction was started by the addition of 100 ng/mL of PMA, stopped by the addition of 0.05% (W/V) DOC at 3 min after PMA addition, and restored 1 min later by the addition of 1 mM NADPH. The amount of O_2^- released from PMA-stimulated hemocytes was calculated as follows: the rate of nmol cytochrome *c* reduced = $\Delta A_{550-540}$/ 19.1 × 10^3/0.455/min/10^7 cells.

Results and Discussion

As shown in all of Figures, O_2 consumption and O_2^- generation in oyster hemocytes were provided as representative data. The representative experiment was selected from the middle one experiment of each set (five experiments).

Effect of DPI on the PMA-stimulated O_2 consumption of oyster hemocytes

A marked increase in O_2 consumption was observed when oyster hemocytes were stimulated with PMA (Fig. 1). And also, addition of 2 μM DPI to PMA-stimulated hemocytes caused a strong inhibition of the O_2 consumption (Fig. 1). The rate of O_2 consumption calculated from the straight slope of the trace (shown a broken line in Fig. 1) was 2.19 μM/min. The pattern of DPI-induced inhibition of O_2 consumption in oyster hemocytes resembled that of rat peritoneal macrophages found by Hancock and Jones (1987). Previous papers demonstrated that DPI is a potent inhibitor of NADPH oxidase from porcine neutrophils (Cross and Jones, 1986) and from rat peritoneal macrophages (Hancock and Jones, 1987). However, treatment of macrophages with DPI showed little inhibitory effect on the respiratory activity of mitochondria (Hancock and Jones, 1987). In this experiment mitochondrial respiration was first blocked by the addition of KCN, therefore, the O_2 consumption of oyster hemocytes, which is inhibited by DPI, seems to undergo a respiratory burst.

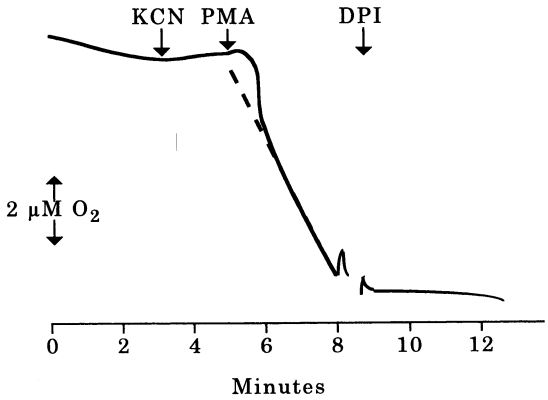

Fig. 1. Effect of DPI on the PMA-stimulated oxygen (O_2) consumption of oyster hemocytes. Arrows indicate the additions of 2 mM KCN, 100 ng/mL of PMA, and 2 μM DPI. The result illustrated is a representative profile of that achieved in five separate experiments.

Influence of DPI on MCLA-enhanced chemiluminescence

PMA-stimulated hemocytes exhibited a relatively strong CL response (Fig. 2). The photocount rapidly increased within the 12-min incubation period, with the peak photocount of 4.67 × 10^5 CPM/2 × 10^6 hemocytes recorded at 18 min after the addition of PMA to the hemocyte suspension. Following this the response declined slowly; the terminal count in this experiment was 3.88 × 10^5 CPM/2 × 10^6 hemocytes at 48 min after PMA addition. The MCLA-enhanced CL produced by PMA-stimulated oyster hemocytes was reduced by 88% in the presence of 2 μM DPI (Fig. 2). In addition, we found that 150 U/mL of Cu, Zn-SOD showed about 80% inhibition against the CL response induced by PMA-stimulated oyster hemocytes (Fig. 2). These results indicate that a large part of the MCLA-enhanced CL response reflects O_2^- generation by the oyster hemocytes, and the O_2^- generation was inhibited by a low concentration of DPI. Further these results are comparable to those reported for *L. stagnalis* and *C. virginica* hemocytes (Adema *et al.*, 1993; Bramble and Anderson, 1999), and for mammalian neutrophils and macrophages (Hancock and Jones, 1987; Hampton and Winterbourn, 1995).

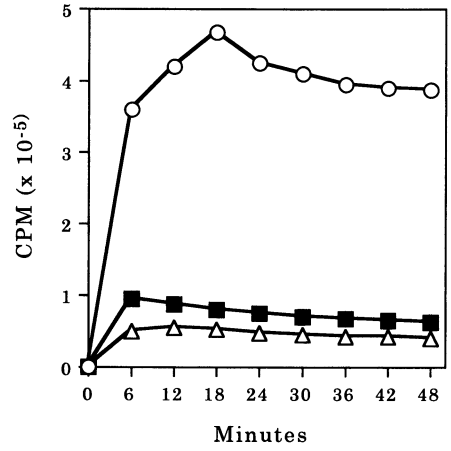

Fig. 2. Effect of DPI on MCLA-enhanced chemiluminescence activity of oyster hemocytes. Oyster hemocytes (2 × 10^6 cells) were stimulated with PMA (100 ng/mL) in the absence (○) or presence (△) of 2 μM DPI, and in the presence of Cu, Zn-SOD (150 U/mL) in the reaction mixture (■).

Detection of O_2^- generation by the reduction of acetylated ferricytochrome c

After the addition of PMA, 1.96 μM/min of ferricytochrome *c* was reduced by 10^7 hemocytes (Fig. 3). The rate of reduction corresponded to that of O_2 consumption (2.19 μM/min), although the two rates could not be measured simultaneously with the same hemocytes sample. These data suggested that most of O_2 consumed in the respiratory burst primarily converted to O_2^- in oyster hemocytes.

This reduction response was promptly stopped by the addition of deoxycholate as an agent for lysing oyster hemocytes (Fig. 3). The addition of exogenous NADPH (1 mM) to the hemocyte lysate resulted in restoration of the reduction of ferricytochrome *c* at 1 min after deoxycholate addition; the rate of cytochrome *c* reduction (NADPH-dependent O_2^- generation) was 0.81 μM of ferricytochrome *c* reduced/min/10^7 hemocytes. Moreover, the reduction response was almost completely inhibited by the addition of 150 U/mL of Cu, Zn-SOD (Fig. 3).

In the previous paper we demonstrated that O_2^- generation in oyster hemocytes was measured by a reduction assay with native ferricytochrome *c*, although 70% of cytochrome *c* reduction was not inhibited by the addition of 150 U/mL of Cu, Zn-SOD, indicating that oyster hemocytes possess nonspecific cytochrome *c* reductases or reducing agents (Takahashi *et al.*, 1993). Kakinuma and Minakami (1978) described that acetylation of ferricytochrome *c* results in a marked decrease in its reducibility by cytoplasmic and microsomal reductases, while reducibility by O_2^- is retained in the experiments using polymorphonuclear leukocytes from guinea pigs. In the present experiments, therefore, we employed acetylated ferricytochrome *c* for the detection

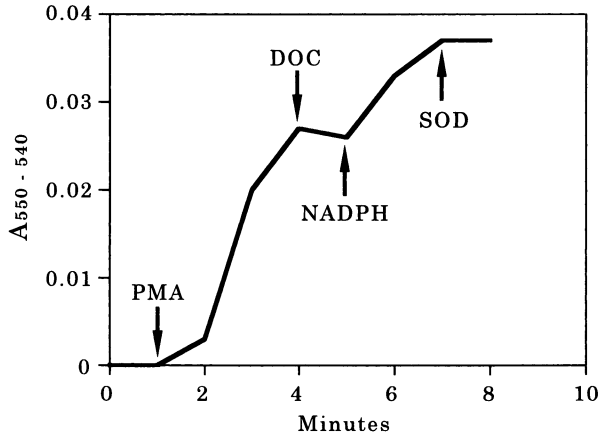

Fig. 3. Detection of O_2^- generation by oyster hemocytes monitored by the reduction of acetylated ferricytochrome *c*. Arrows indicate the additions of 100 ng/mL of PMA, 0.05% (W/V) deoxycholate (DOC), 1 mM NADPH, and 150 U/mL of Cu, Zn-SOD. The result shown in a representative profile of that achieved in five separate experiments.

of O_2^- generated in intact and lysed oyster hemocytes. As a result, we demonstrated that O_2^- generation by oyster hemocytes lysate seems to be related to an activation or a reconstitution of cellular components dependent on NADPH. Baier-Anderson and Anderson (1997) showed that both NADPH production and O_2^- generation by *C. virginica* hemocytes significantly decreased after exposed *in vitro* to a biocide pentachlorophenol. These data suggest NADPH is a required element in the generation of O_2^- by *C. virginica* hemocytes.

The present study shows that in oyster hemocytes, PMA-induced O_2 consumption and O_2^- generation are associated with the respiratory burst, but not with mitochondrial respiration. Both the O_2 consumption and O_2^- generation are strongly inhibited by the addition of a low concentration of DPI, a potent inhibitor of NADPH oxidase. Moreover, we show that O_2^- generation by oyster hemocytes lysate depends on the presence of NADPH in the reaction mixture. Although the present study does not provide molecular analysis of components of NADPH oxidase and kinetics of oxygen metabolism during the respiratory burst, we conclude that NADPH oxidase-like activity occurs in the Pacific oyster hemocytes.

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