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Measurement of membrane fluidity of rockfish (*Sebastes schlegeli*) phagocytes during the respiratory burst using fluorescence polarization assay

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The change of membrane fluidity in rockfish *(Sebastes schlegeli)* phagocytes during respiratory burst was investigated. Fluorescence polarization (FP) was used as a measure of membrane fluidity, and 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was used as a fluorescent probe. The significantly higher FP values in phagocytes stimulated with zymosan or phorbol myristate acetate (PMA) than unstimulated control phagocytes suggests that membrane fluidity of phagocytes is decreased during the respiratory burst. The faster decrease of FP value in PMA stimulated phagocytes than in zymosan stimulated phagocytes may be due to bypass of the receptor-mediated stages of functional modulation, which is needed in zymosan stimulated phagocytes.

Key words : Membrane fluidity, Phagocyte, Respiratory burst, Fluorescence polarization

Reactive oxygen species (ROS) generation by respiratory burst is increasingly recognized as an important defense mechanism (Babior *et al.*, 1973; Secombes and Fletcher, 1992; Sakai, 1999). This respiratory burst is triggered by the NADPH oxidase system, which is activated following the perturbation of the plasma membrane during phagocytosis or following the interaction between the cell surface and a variety of agents (Rossi *et al.*, 1985). The biochemical events following NADPH oxidase activation are multiple and complex, involving a number of processes which take place at the level of cell surface, plasma membrane and cytosol (Rossi, 1986; Lambeth, 1988; Baggiolini and Wymann, 1990). The steady state fluorescence polarization techniques using various fluorescence probes have proved a reliable method for the characterization of membrane fluidity in many studies (Stubbs, 1983; Bashford, 1994). It is shown that fluorescence anisotropy is proportional to the order of molecular packing and inversely proportional to membrane fluidity, thus directly usable for the characterization of the properties of membranes (Molitoris, 1987).

Several modifications of the plasma membrane fluidity of phagocytes have been described in association with the respiratory burst in mammals (Valentino *et al.*, 1988; Fiorini *et al.*, 1990; Lo Presti *et al.*, 2002). In fish, however, there is no available information on the change of phagocyte membrane fluidity in relation to respiratory burst. In the present study, therefore, we investigated the change of membrane fluidity in rockfish (*Sebastes schlegeli*) phagocytes during stimulation with zymosan and phorbol myristate acetate (PMA) known to activate respiratory burst. Fluorescence polarization (FP) was used as a measure of membrane fluidity, and 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), which goes

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to the lipid-water interface region of the plasma membrane (Illinger *et al.*, 1995), was used as a fluorescent probe.

Juvenile rockfish (*Sebastes schlegeli*), weighing 80-100 g, obtained from a local fish farm, was kept in 500 L fiberglass tanks containing filtered, aerated sea water at 20±2℃. Fish were acclimated to these conditions for at least 2 weeks before use, and fed commercial rockfish pellets. Fish were anaesthetized with tricaine methanesulfonate (MS222; Sigma, St. Louis, MO). The head-kidney was extracted by ventral incision and transferred to L-15 medium (Sigma) supplemented with 2% foetal calf serum (FCS; Sigma), heparin (10 units/㎖, Sigma), penicillin (100 ㎍/㎖, Sigma) and streptomycin (100 U/㎖, Sigma). The cell suspensions obtained by forcing the organ through a nylon mesh were layered over a 34/51% Percoll (Sigma). After centrifugation at 400 x g for 30 min at 4℃, the phagocyte enriched interphase was collected and washed three times with L-15 medium. Then, the cells were resuspended in the culture medium, and dispensed into flat-bottomed 96-well plates. After 2 h at 20℃, wells were washed with culture medium to remove non-adherent cells. The remained phagocytes were detached from the plates after incubating for 1 h at 4 ℃. The cell viability was examined with tryphan blue exclusion, and evaluated to be greater than 95%. The number of phagocytes were adjusted to 1 × 10^6 cells/㎖, and were labeled with 1 M of 1-(4-trimethylaminophenyl)-6-phenyl-1, 3, 5-hexatriene (TMA-DPH; Sigma) at 25℃ for 4 min. The labeled phagocytes were added into a black 96-well plate (Greiner Bio-One Inc., USA), and were stimulated with zymosan (Sigma) or phorbol myristate acetate (PMA; Sigma). Wells without the stimulants were used as controls and wells with only Hank's balanced salt solution (HBSS; Sigma) were designated as blanks. The fluorescence polarization was measured by a Polarion instrument (TECAN Austria GmbH, Austria). The excitation wavelength was 360 nm and the emission wavelength was 435 nm. All assays were done in triplicate. Readings were automatically recorded at 5, 15, 25, 35, 45, 55 and 65 min in millipolarization units (mP). The Student's *t*-test was used to determine statistical differences. The results were considered significant when $P<0.05$.

In results, fluorescence polarization (FP) values were significantly higher in zymosan stimulated phagocytes than in PMA stimulated and non-stimulated control phagocytes at all assayed times (Fig. 1). Phagocytes stimulated with PMA showed significantly higher FP values than control phagocytes at 5, 15, 25 and 35 min. Phagocytes stimulated with PMA showed faster decrease of FP values than phagocytes stimulated with zymosan (Fig. 1).

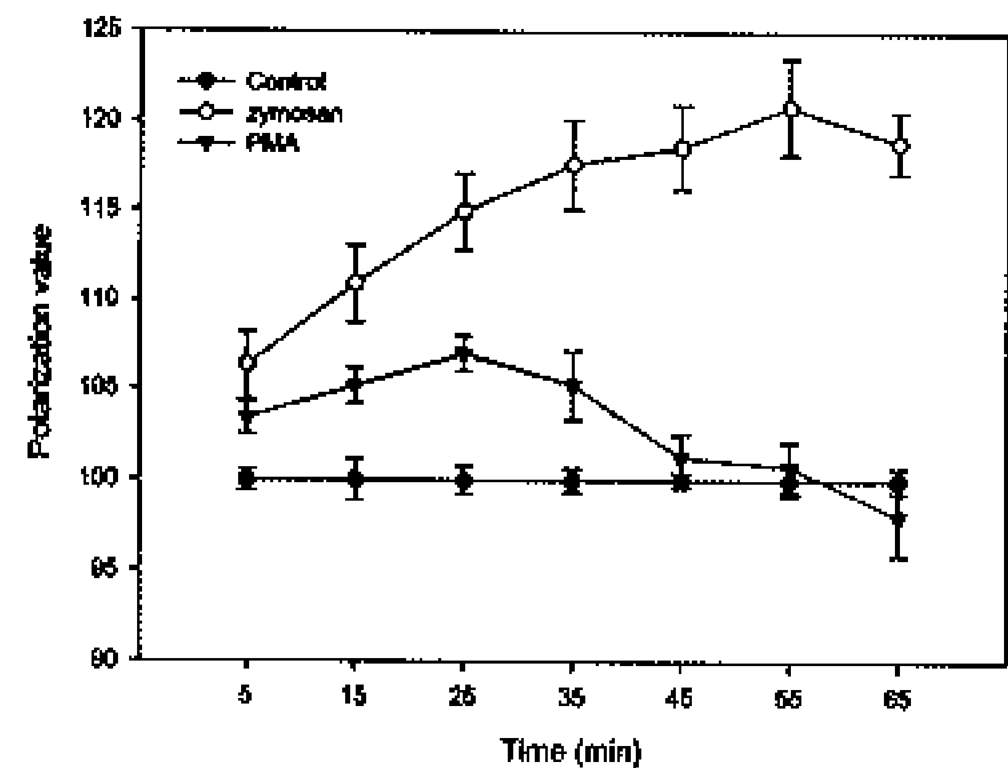

Fig. 1. Fluorescence polarization value of TMA-DPH in phagocytes of rockfish (*Sebastes schlegeli*) stimulated with zymosan or phorbol myristate acetate(PMA). points represent mean values ± standard deviation.

Cell membranes comprise a lipid bilayer consisting mainly of phospholipids and cholesterol. The functionality of proteins embedded within this environment is essentially affected by the physicochemical properties of the surrounding membranes. Often the physical state of membranes is characterized by fluidity, which is defined as the relative

motional freedom of lipid molecules within the bilayer (Bashford, 1994). In the present study the fluorescence polarization of TMA-DPH was used for the characterization of the influence of different stimulants on the fluidity of rockfish phagocyte membrane during respiratory burst. The TMA-DPH fluorescent probe has been used intensively in studying membrane structure and fluidity because of its advantageous structural and photophysiological properties (Lentz, 1989; VanGinkel, 1989). It displays an amphipathic character revealed by partition equilibrium between water and membranes. This partition results in fluorescence intensity being proportional to the cell membrane surface changes or for monitoring endocytosis or intracellular membrane traffic (Illinger *et al.*, 1995). Moreover, TMA-DPH is incorporated into the membrane but remains at the lipid-water interface region because of its cationic residue for at least 30 min allowing evaluation of the membrane response to stimuli (Kuhry *et al.*, 1983). In this study, the significantly higher fluorescent polarization (FP) values in phagocytes stimulated with zymosan or PMA than unstimulated control phagocytes suggests that membrane fluidity of phagocytes is decreased during the respiratory burst. Fiorini *et al.* (1990), also, reported that membrane fluidity of human polymorphonuclear leukocytes was increased by activation of respiratory burst. From the present result, it can be conjectured that excess of reactive oxygen species (ROS) generated by phagocytes may damage biologically important macromolecules, especially membrane lipids, which are particularly vulnerable to peroxidation, and leads to alterations in membrane fluidity and permeability.

The faster decrease of FP values in PMA-stimulated phagocytes than in zymosan-stimulated phagocytes in this study suggests that PMA has a shorter stimulatory potential of respiratory burst compared to zymosan. Zymosan, as a potent stimulant of phagocytosis, binds to complement and immunoglobulin receptors CR3 and Fc receptors on the cell surface, leading to phagocytosis, activation of NADPH-oxidase, degranulation of specific and azurophilic granules and formation of phagolysosomes (Bainton, 1973). In contrast, PMA is a soluble stimulant, activates NADPH-oxidase and degranulation via activation of protein kinase C in place of diacylglycerol (Morel *et al.*, 1991). Therefore, the faster decrease of FP value in PMA stimulated phagocytes in the present result may be due to bypass of the receptor-mediated stages of functional modulation, which is needed in zymosan stimulated phagocytes.

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