

Comparison between Lucigenin- and Luminol-dependent Chemiluminescence Responses of Rockfish (*Sebastes schlegeli*) Head Kidney Phagocytes

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Lucigenin (Lg)- and luminol (Lm)-dependent chemiluminescence (CL) was used to compare the respiratory burst of rockfish (*Sebastes schlegeli*) phagocytes after stimulation with phorbol myristate acetate (PMA). To establish which reactive oxygen species (ROS) contributes to the observed CL, the modulators of ROS metabolism, such as superoxide dismutase (SOD), catalase, and sodium azide (NaN_3) were used. Although LgCL responses were inhibited significantly by the addition of either SOD or catalase, in comparison to the control, significantly lower LgCL responses were recorded by SOD than catalase. LmCL also showed significantly decreased responses by the addition of SOD and catalase. However, there were no statistical differences in CL responses between SOD and catalase additions. More profound and significant decrease of LmCL responses were recorded by simultaneous addition of SOD and catalase. Sodium azide markedly enhanced LgCL responses, while it significantly inhibited LmCL responses. These results indicate that LgCL and LmCL can be used to measure extracellular O_2^- production and myeloperoxidase (MPO)-mediated ROS production in fish phagocytes, respectively. Furthermore, LmCL can be used for analyzing intracellular ROS production by simultaneous addition of both SOD and catalase.

Key words: Fish phagocyte, Lucigenin, Luminol, Chemiluminescence, *Sebastes schlegeli*

Introduction

The ability of phagocytes to fulfill their role in the defense against invading microorganisms is highly dependent on reactive oxygen species (ROS) generated during the respiratory burst (Dinauer, 1993; Neumann et al., 2001; Vazzana et al., 2003). Superoxide is generated by one electron reduction of molecular oxygen and is dismutated into hydrogen peroxide, which can be converted to hydroxyl radical or metabolized to hypochlorite by myeloperoxidase in leukocytes (Allen et al., 1972; Babior, 1984).

Since the chemiluminescence (CL) response measures the photon emission resulting from ROS formation, the measurement of CL activity of phagocytes is an assay currently used to study the kinetics of the respiratory burst in mammals (Saniabadi and Nakano, 1993; Nathan and Shiloh, 2000) as well as in fish (Scott and Klesius, 1981; Neumann et al.,

2001). Although lucigenin-dependent chemiluminescence (LgCL) and luminol-dependent chemiluminescence (LmCL) have been used to measure various metabolic stages of ROS, the comparative contributions of ROS metabolites to LgCL and LmCL have not been fully defined in respiratory burst of fish phagocytes.

We analyzed LgCL and LmCL responses of rockfish (*Sebastes schlegeli*) phagocytes in the presence or absence of the modulators of ROS metabolism such as superoxide dismutase (SOD), catalase and sodium azide (NaN_3) in order to establish which ROS contributes to the observed CL.

Materials and Methods

Fish

Juvenile rockfish (*Sebastes schlegeli*), weighing 80-100 g, obtained from a local fish farm, was kept in 500 L fiberglass tanks containing filtered sea water at $20 \pm 2^\circ\text{C}$ with mild aeration. Fish were acclimated

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to these conditions for at least 2 weeks before use, and fed commercial rockfish pellets.

Isolation of head kidney phagocytes

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 mL⁻¹, Sigma), penicillin (100 µg mL⁻¹, Sigma) and streptomycin (100 U mL⁻¹, 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 g for 30 min at 4°C, the phagocyte enriched interphase was collected and washed three times. Then, the cells were resuspended in culture medium, and dispensed into flat-bottomed 96-well plates. After 2 h at 20°C, wells were washed with culture medium to remove non-adherent cells. The remained phagocytes were detached from the plates by incubating for 1 h at 4°C. The cell viability was examined with trypan blue exclusion and evaluated to be greater than 95%. The number of phagocytes were adjusted to 1×10⁶ cells mL⁻¹.

Chemiluminescence (CL) assay

The reactive oxygen species (ROS) produced by stimulated phagocytes was quantified using an automatic photoluminometer (Bio-Orbit 1251, Finland). The chemiluminogenic probes lucigenin (bis-*N*-methylacridinium nitrate, Sigma) and luminol (5-amino-2,3-di-hydro-1,4-phthalazinedione, Sigma) were dissolved in dimethyl sulphoxide (DMSO, Sigma) to stock solutions and diluted in Hank's balanced salt solution (HBSS, Sigma) to final assay concentrations of 0.1 mM and 5 mM, respectively. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) at 1 µg mL⁻¹. Each test cuvette contained 0.7 mL of either lucigenin or luminol, 0.4 mL of cell suspension and 0.1 mL of PMA, which was added just prior to measurement. The measurements were made for 1 h. All assays were performed in triplicate and the intensity of CL responses was expressed as the percentage of peak value of CL supplemented with ROS modulators/peak value of CL supplemented HBSS alone (control).

ROS modulators

Superoxide dismutase (SOD, Sigma) and Catalase (Sigma) were diluted in HBSS to final concentrations of 40 U and 2 U, respectively. Sodium azide (NaN₃,

Sigma) was diluted with HBSS at 1 mM. The solutions of these reagents were added to phagocyte suspensions prior to cell stimulation.

Statistical analysis

The Student's t-test was used to determine statistical differences. The results were considered significant when P<0.05.

Results

Although LgCL responses were inhibited significantly by addition of either SOD or catalase compared to control, significantly lower LgCL responses were recorded by SOD than catalase (Fig. 1). LmCL also showed significantly decreased response by addition of SOD and catalase. However, there were no statistical differences in CL responses between SOD and catalase additions. More profound and significant decrease of LmCL responses was recorded by simultaneous addition of SOD and catalase (Fig. 1).

Sodium azide markedly enhanced LgCL response, while it significantly inhibited LmCL responses (Fig. 2).

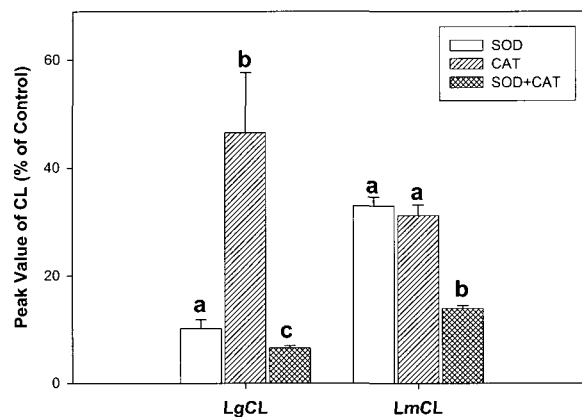


Fig. 1. Effects of superoxide dismutase (SOD), catalase (CAT) and SOD plus CAT on lucigenin-dependent chemiluminescence (LgCL) and luminol-dependent chemiluminescence (LmCL) of rockfish (*Sebastes schlegeli*) head kidney phagocytes. Bars represent mean±standard deviation of triplicate assays. Different letters on the bar represent statistical significance at P<0.05.

Discussion

In chemiluminescence (CL), luminol is often described as permeating cells and reacting with different ROS, both intracellular and extracellular,

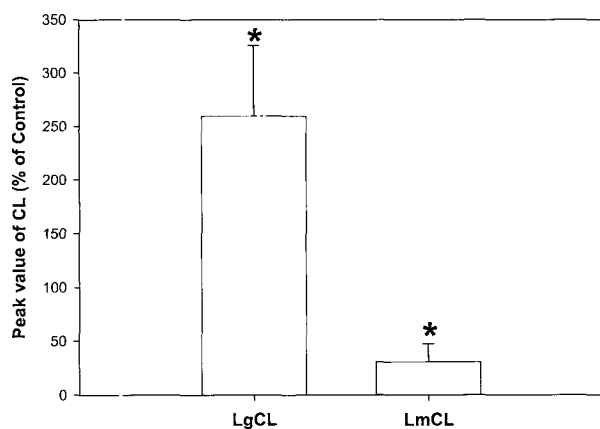


Fig. 2. Effects of sodium azide on lucigenin-dependent chemiluminescence (LgCL) and luminol-dependent chemiluminescence (LmCL) of rockfish (*Sebastes schlegeli*) head kidney phagocytes. Bars represent mean \pm standard deviation of triplicate assays, and asterisks denote significant difference ($P < 0.05$) from control value.

whereas lucigenin is known to react specifically with extracellular superoxide anion and does not permeate cells (Briheim et al., 1984; Rest 1994; Caldefie-Chézet et al., 2002). The most direct way to determine the role of superoxide anion and hydrogen peroxide in the CL reaction is to investigate the effects of specific scavengers or enzyme inhibitors such as superoxide dismutase (SOD; catalyses the dismutation of O_2^- to H_2O_2) and catalase (reduces H_2O_2 to water). In the present study, the significantly higher inhibition of LgCL by SOD than by catalase suggests that LgCL is more sensitive to extracellular O_2^- than H_2O_2 . In contrast, inhibition of LmCL by SOD and catalase was not different significantly, and this indicates that LmCL is sensitive to both O_2^- and H_2O_2 . Superoxide anion is a critical intermediate involved in the reaction pathway leading to LmCL. Therefore, inhibition by SOD of LmCL appears to result from their scavenging O_2^- generated from the luminol radical. Similarly, LmCL of peripheral blood phagocytes from channel catfish (Scott et al., 1985) and human neutrophils (Allred et al., 1980) was almost completely blocked by SOD, and human polymorphonuclear leukocyte CL was decreased partially in the presence of SOD (Jancinovic et al., 2001). In this work, exogenous SOD inhibited the CL response of rockfish head kidney phagocytes. One explanation of this CL inhibition by SOD is that the leukocytes possess two hydrogen peroxide detoxification systems, namely catalase and/or glutathione peroxidase. The addition of ex-

ogenous SOD, which produces H_2O_2 from superoxide, if coupled with endogenous catalase and/or glutathione peroxidase, would decrease the CL amplified by luminol (Vazzana et al., 2003). In fact, SOD and catalase added simultaneously in the present study inhibited LmCL synergistically. Macromolecular proteins like SOD and catalase can only scavenge extracellularly released metabolites, and luminol, which is more lipophilic than lucigenin, strongly interacts with ROS on both sides of the cell membrane (Allen, 1986). Therefore, in the present study, LmCL measured by simultaneous addition of SOD and catalase is suggestive of the intracellular production of ROS.

Enhanced LmCL appears to depend largely on the myeloperoxidase (MPO)- H_2O_2 -Cl system in cells (McNally and Bell, 1996). The present study confirmed this by the use of fish phagocytes, since sodium azide (NaN_3) markedly decreased LmCL. MPO is a major component of the bactericidal armamentarium of phagocytes, due to its capacity to catalyze the production of hypochlorous acid (HOCl), a powerful oxidant (Hyslop et al., 1995). In human, neutrophils isolated from donors with MPO deficiency produce very low levels of LmCL despite a pronounced production of O_2^- and H_2O_2 (Aniansson et al., 1984; Kusenbach and Rister, 1985). Therefore, the present results suggest that MPO-derived metabolites are responsible for the excitation of luminol. On the other hand, in the present results, LgCL response was enhanced by NaN_3 . This suggests the accumulation of superoxide anions leads to the excitation of LgCL, since NaN_3 inhibits both catalase and MPO activities, and the consumption of H_2O_2 is blocked.

In conclusion, the present results indicate that LgCL and LmCL can be used for measuring extracellular O_2^- production and MPO-mediated ROS production in fish phagocytes, respectively. Furthermore, LmCL can be used for analyzing intracellular ROS production by simultaneous addition of both SOD and catalase.

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