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Effects of scavengers for active oxygen species on photoresponse and photodynamic damages to a pigmented protozoan, *Blepharisma*

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ABSTRACT

In the absence of O_2, the step-up photophobic response of *Blepharisma japonicum* is suppressed. To evaluate the possibility that reactivated oxygen species (ROS) produced by blepharismin photosensitization are involved in the primary transduction for the step-up photophobic response of *B. japonicum*, the effects of OH radical scavengers and the singlet oxygen quencher on the photophobic response were examined. The reagents used in the present assays hardly suppressed the step-up photophobic response, even though OH radical scavengers were rescued from the photodynamic killing of the cells. Alternatively, in the presence of an electron acceptor, the latency of the step-up photophobic response was prolonged. In addition, oxyblepharismin fluorescence was quenched slightly in the presence of free amino acids. These results support a hypothesis proposed by Lenci's research group, that electron transfer from the first excited singlet state of blepharismin (or oxyblepharismin) to associated proteins may be involved in the primary phototransduction for the step-up photophobic response.

Keywords: *Blepharisma*; phototransduction; reactivated oxygen species

INTRODUCTION

The ciliated protozoan *Blepharisma japonicum* has a number of pigment granules in a subpellicular region containing a pink quinoid pigment (Maeda *et al*. 1997; Checcucci *et al.* 1997) called blepharismin (Giese 1973). When solution containing blepharismin or its oxidized form (oxyblepharismin) is exposed to light, hydroxyl radicals (Kato and Matsuoka 1995; Kato *et al.* 1996) and singlet oxygen (Checcucci *et al.* 2001) are generated by pigment photosensitization. In this case, superoxide anion is not produced (Kato *et al.* 1996). A primary role of the pigment is to provide a chemical defense by its light-independent toxic effect against predatory protozoans (Miyake *et al*. 1990; Harumoto *et al*. 1998; Noda *et al*. 1999). The toxic effect of blepharismins is enhanced by light irradiation because of pigment photosensitization (Giese 1973; Kato and Matsuoka 1995; Harumoto *et al*. 1998). However, a strong light causes lethal damage even to *Blepharisma* itself (Giese 1946), although *Blepharisma* has acquired an antioxidative protection system by the light-induced expression of genes homologous to the glutathione *S*-transferase gene (Takada *et al*. 2004; Takada and Matsuoka in press). Therefore, *Blepharisma* avoids illuminated areas by a temporary backward movement achieved by reversing the direction in which the cilia beat (Kraml and Marwan 1983; Matsuoka 1983). This response, called the step-up photophobic response, is elicited usually 1~2 sec after light perception. Blepharismin, which may be associated with 38 kDa (Gioffré *et al*. 1993) or 200 kDa protein (Matsuoka *et al*. 1993; Matsuoka *et al*. 2000), are believed to function as the photoreceptors responsible for the step-up photophobic response (Scevoli *et al*. 1987; Matsuoka *et al*. 1992; Checcucci *et al*. 1993).

Fluorescence quenching of oxyblepharismin occurs in the presence of adequate electron acceptors (Angelini *et al*. 1998). The pigment associated with the native apoprotein (100~200 kDa) shows a short-lived absorption feature and an ultrafast fluorescence decay as compared with free pigment (Plaza *et al*. 2005). Based on these findings, Lenci's research group proposed that electron transfer from the first-excited singlet state of blepharismin (oxyblepharismin) to a neighboring protein acceptor may activate a photosignaling pathway (Plaza *et al*. 2005). On the other hand, the fact that the lack of O_2 blocks the step-up photophobic response of *Blepharisma* (Matsuoka *et al*. 1995) implies that activated oxygen species produced by blepharismin photosensitization may activate apoproteins. In another ciliated protozoan, *Loxodes*, oxygen radicals have been suggested to act in phototransduction, based on the fact that *Loxodes* does not respond to light in the absence of O_2 (Fenchel and Finlay 1986). On the other hand, it has been reported that neither crocetin (a singlet oxygen quencher) nor 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), a spin-trapping reagent for activated oxygen species, suppresses the step-up photophobic response (Checcucci *et al*. 1991; Kida *et al*. 2001). The present study aimed to further evaluate the possibility that OH radicals or singlet oxygen might be responsible for the step-up photophobic response by using scavengers for OH radicals such as DCFH (Myhre *et al*. 2003), ascorbic acid (Bagchi *et al*. 1997), 5-HTP (Keithahn and Lerchl 2005), edaravone (Watanabe *et al*. 2004), curcumin (a quencher for singlet oxygen) (Das and Das 2002), and active hydrogen water (electrolyzed-reduced water, an inactivator for both ROS) (Shirahata *et al*. 1997). In addition, whether the cells are rescued from the photodynamic killing in the presence of ROS inactivators was examined.

MATERIALS AND METHODS

Cell Culture

B. japonicum was cultured at 23°C in the dark in a 0.1% (w/v) infusion of dried cereal leaves containing bacteria (*Enterobacter aerogenes*) as a food organism. Bacteria (*E. aerogenes*) were cultured on agar plates containing 1.5% agar, 0.5% polypepton, 1% meat extract, and 0.5% NaCl. The cultured cells were collected by centrifugation (150 xg, 1 min), subsequently suspended in a standard saline solution containing 1 mM $CaCl_2$, 1 mM KCl, and 5 mM Tris-HCl (pH 7.2), and kept for 1~2 days.

Chemicals

2',7'-dichlorofluorescin diacetate (DCFH-DA), diferuloylmethane (curcumin), 1,4-benzoquinone, and hydroquinone were dissolved in 100% dimethyl sulfoxide (DMSO) for the stock solutions. These stock solutions were diluted by 500-fold in a standard saline solution. 5-Hydroxytryptophan (5-HTP) and 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone) were dissolved directly in the standard saline solution. The standard saline solution containing active hydrogen (H_4O) was prepared by dissolving the solutes in active hydrogen water. The chemicals tested in the present assays were obtained from Wako Pure Chemical Industries except for DCFH-DA (Sigma-Aldrich), curcumin (Sigma), edaravone (Aldrich), active hydrogen water (Ryukyufront), and 1,4-benzoquinone (Tokyo Chemical Industry). Photodynamic killing and the step-up photophobic response of *Blepharisma* were examined after the cells were incubated for 10 min in solutions containing chemicals.

Assays for photodynamic action and photoresponse

Photodynamic killing of *Blepharisma* was observed after the cells were exposed to white light (3,000 W/m^2) for 10 min using a fiber-optic light source (Nikon), and the viability was expressed as a percentage of the total number of tested cells (100 cells). In this case, motionless or burst cells were counted as killed cells. The rates of step-up photophobic response were expressed as the percentage of the total number (50-100

cells) showing the response 5 sec after the onset of light stimulation (580-nm light, 8 x 10^{18} quanta/m^2s). In the assays of photodynamic killing of the cells and the step-up photophobic response (Fig. 2, A-1~F-1, A-2~F-2; Fig. 3), columns and attached bars indicate the means of six identical measurements and SE, respectively. In the assays of latency (Fig. 2, A-3~F-3), the columns and attached bars correspond to the means and SE obtained from 30 measurements (30 cells responding within 5 sec after light stimulation), respectively.

Visualization of OH radical generation

To stop ciliary movement of *Blepharisma*, an equal volume of the standard saline solution containing 2 mM $NiCl_2$ was mixed with the cell suspension that had been adapted for 2 hr in dark. Thereafter, 2 μl of 10 mM stock solution of DCFH-DA dissolved in DMSO was added to 1 ml of the suspension of Ni^{2+}-paralyzed cells, and was kept for 30 min in the dark. The cells were rinsed twice with standard saline solution containing 1 mM $NiCl_2$, placed on a slide glass, and then exposed to 480-nm light (bandwidth, 470-490 nm; 5 x 10^{20} quanta/m^2s) for 30 sec. The DCF fluorescence was obtained by an excitation light of 480 nm (bandwidth, 470-490 nm), and fluorescence ranging from 515 to 550 nm was detected using a filter (Olympus, BA515-550). The fluorescence micrograph images were acquired by a digital camera (Nikon, Coolpix 4500) attached to a microscope (Olympus, BX50).

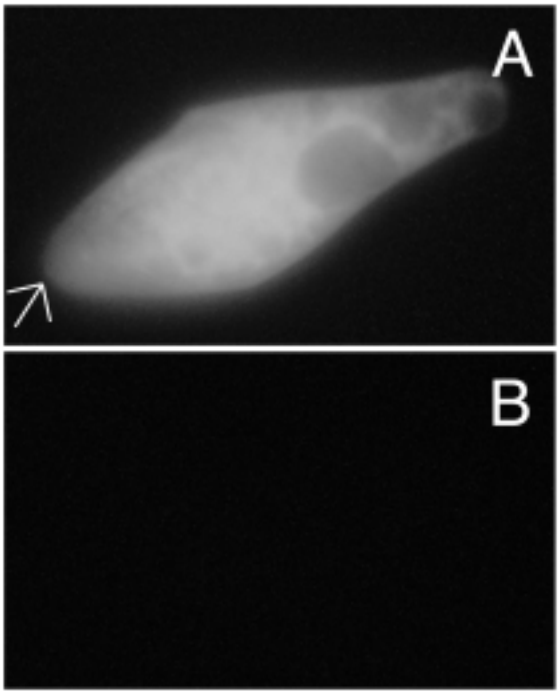

Fig. 1. Fluorescence micrographs of cells into which DCFH-DC was introduced. (A) *Blepharisma* exposed to 480-nm light (5 x 10^{20} quanta/m^2s) for 30 sec. Arrow, anterior end of the cell. (B), *Blepharisma* kept in dark.

Quenching assays of oxyblepharismin fluorescence by amino acids

To extract blepharismin, cells were collected (150 xg, 1 min) and suspended in acetone. After 1 min of extraction, cells were sedimented (150 xg, 5 min) and the supernatant was decanted. The supernatant containing pigment was dried with a rotary evaporator (Rotavapor, Shibata, Japan), and the dried blepharismin was exposed to white light (40 W/m^2) overnight to convert oxyblepharismin. The sample was dissolved in a small amount of acetone for the application to normal-phase TLC plates with silica gel 60A

LK6F (Whatman) and then was developed with a 2:1 mixture of ethyl acetate and acetone. Purified oxyblepharismin (12 μg/ml each) was dissolved in a solution containing 10 mM Tris-HCl (pH 7.2), 10 mM sodium cholate, and 10 mM or 1 mM of substances tested for a fluorescence quencher (10 mM 1,4-benzoquinone, or 10 mM amino acids except for 1 mM Glu, and 1 mM Lys). Fluorescence emission spectra, obtained by excitement with a 490-nm light, was recorded by a spectrofluorophotometer (Shimadzu, RF-510).

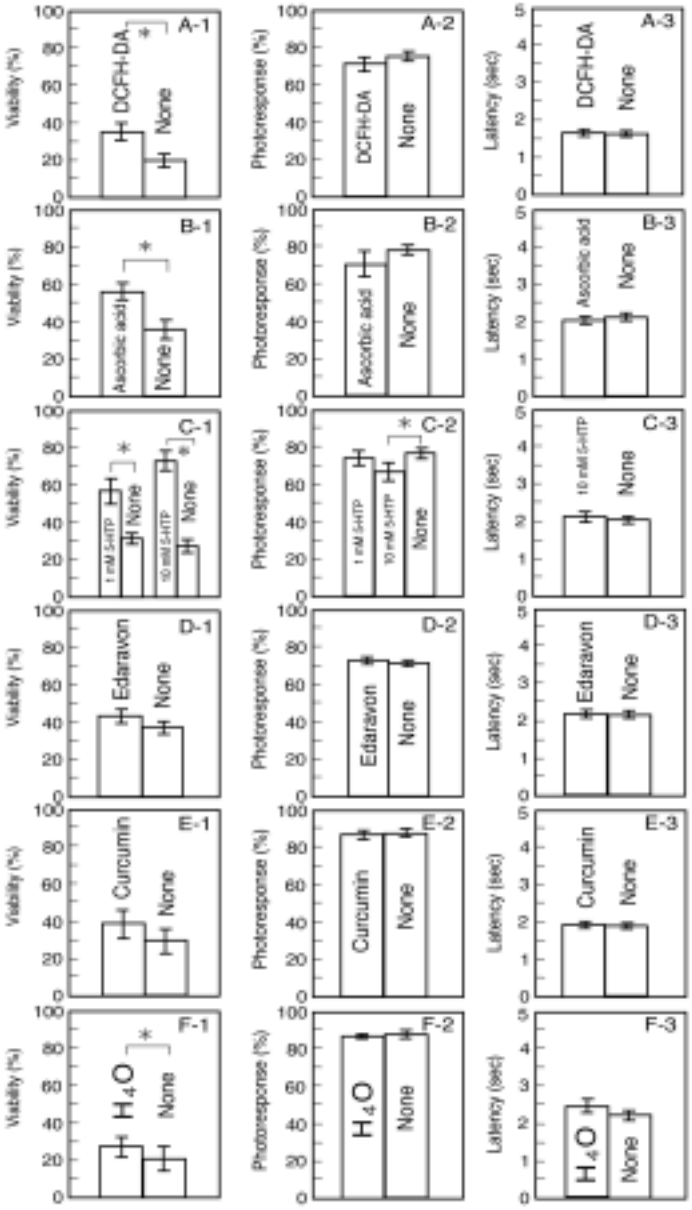

Fig. 2. Effects of ROS scavengers on photodynamic killing (A-1~F-1), on the rate of step-up photophobic response (A-2~F-2), and on the latency (A-3~F-3) of the step-up photophobic response of *Blepharisma*. (A) 20 μM DCFH-DA, (B) 10 mM ascorbic acid, (C) 1 mM and 10 mM 5-HTP, (D) 100 μM edaravone, (E) 2 μM curcumin, (F) active hydrogen water (H_4O). Columns labeled 'None' indicate the viability of the cell suspended in the standard saline solution without ROS scavengers or quenchers. *, significantly different from each other ($p < 0.05$, Mann-Whitney test).

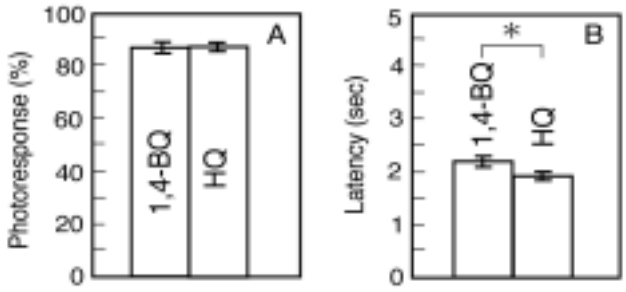

Fig. 3. Effects of electron acceptor (1 μM 1,4-benzoquinone) on the rate (A) and latency (B) of step-up photophobic response of *Blepharisma*. As a control, 1 μM hydroquinone (HQ) was used. *, significantly different from each other ($p < 0.05$, Mann-Whitney test).

RESULTS AND DISCUSSION

Upon introduction to a cell interior, 2',7'-dichlorofluorescin (DCFH) is converted to the fluorescent form (DCF) when it is oxidized by ROS, especially by OH radicals (Myhre *et al*. 2003). When a *Blepharisma* cell to which DCFH-DA had been introduced was exposed to light (480 nm, 5 x 10^{20} quanta/m^2s) for 30 sec, a prominent fluorescence was detected (Fig. 1A). On the other hand, the cell kept in the dark did not emit fluorescence at all (Fig. 1B). These results indicate that light exposure of *Blepharisma* produces a serious oxidative environment due to ROS, especially OH radicals.

Figure 2 shows the effect of ROS on photodynamic killing (A1~F1), the rate of step-up photophobic response (A-2~F-2), and the latency (A-3~F-3) of that response. The concentrations of the chemicals employed in the present assays (Figs. 2 and 3) were the upper limits at which most of the cells would be viable and show normal motility. Except for edaravone, most of the scavengers for OH radicals (DCFH, ascorbic acid, 5-HTP) showed a marked rescue effect from photodynamic killing (significantly different from the viability in the absence of scavengers) (Mann-Whitney test, $p < 0.05$). Curcumin, a singlet oxygen quencher, did not show a significant rescue effect. Active hydrogen water (H_4O) slightly rescued cells from photodynamic killing (Mann-Whitney test, $p < 0.05$). On the other hand, the ROS scavengers and quencher hardly affected the step-up photophobic response (Fig. 2, A-2~F-2; A-3~F-3), although a much higher concentration of 5-HTP suppressed the response (Mann-Whitney test, $p < 0.05$).

Visualization of OH radical generation by DCFH-DA (Fig. 1) and of the rescue effects by the OH radical scavengers from photodynamic killing (Fig. 2) indicate that radical scavengers evidently approach the pigment granules (Matsuoka *et al*. 2000; Matsuoka and Kotsuki 2001) where OH radicals are generated. Nevertheless, the radical scavengers hardly affected the step-up photophobic response. This implies that ROS are not involved in photosignal transduction for the step-up photophobic response. Although ROS produced by pigment photosensitization mediates the activation of blepharismin-associated putative photoreceptor protein, a specific protein residue cannot be oxidized, because a number of O_2 may occur in various positions in the vicinity of the protein. Such non-specific oxidation of protein residues seems to frequently cause disordered conformational changes of the protein. In conclusion, it seems unlikely that ROS could be involved in the primary phototransduction for the step-up photophobic response.

The question then arises, Why is the step-up photophobic response of *Blepharisma* suppressed in the absence (below 0.5 mg/l) of O_2 (Matsuoka *et al*. 1995) In the absence of O_2, the velocity with which cells swim forward increased prominently in the dark (Kida *et al*. 2001). In other ciliates, a drop in intracellular O_2

concentration elicits acceleration in forward swimming velocity (Cronkite and Van Den Brink 1981). It is possible that *Blepharisma* may sense a drop in O_2 concentration and suppress the step-up photophobic response concomitantly with forward swimming acceleration (personal communication with Dr. G. Checcucci).

By virtue of the fact that a quenching of oxyblepharismin fluorescence occurs in the presence of electron acceptors such as 1, 4-benzoquinone, Lenci's research group has proposed that the electron transfer from the first-excited singlet state of the pigment to a certain amino acid residue of the blepharismin-associated protein activates the photosignaling pathway (Angelini *et al*. 1998). The introduction of electron acceptors into the cell interior, therefore, is expected to suppress the step-up photophobic response. As shown in Fig. 3, the latency of the step-up photophobic response was longer in the presence of 1,4-benzoquinone than in the presence of hydroquinone ($p < 0.05$, Mann-Whitney test).

If the electrons from blepharismin (oxyblepharismin) should be efficiently transferred to neighboring amino acid residues in the microenvironment of the protein pocket, it is expected that electron transfer from the pigment may occur even in pigment solution containing free amino acids. Actually, most amino acids quenched oxyblepharismin fluorescence, although specific and strong quenching effect by amino acids were not observed; the values of F_0/F, where F_0 and F are the fluorescence intensities without and with the quenchers, respectively, ranged from 1.0 to 1.17 (data not shown) in the presence of each amino acid, and the value is 3.75 in the presence of 10 mM 1,4-benzoquinone. In conclusion, the present results support the hypothesis that electron transfer from blepharismin to certain amino acid residues of the blepharismin-embedded protein pocket may activate the phototransduction pathway leading to the step-up photophobic response.

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