Mercury-induced oxidative stress in marine phytoplankton *Tetraselmis tetrathele* (Prasinophyceae)

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ABSTRACT

Mercury-induced oxidative stress was observed in the cells of marine phytoplankton *Tetraselmis tetrathele* (Prasinophyceae) using the fluorescent probes 2' 7'-dichlorofluorescin diacetate (DCFH-DA) and fluorescein diacetate (FDA). When the cells were exposed to 1 μM of $HgCl_2$, the fluorescence intensity of 2' 7'-dichlorofluorescin (DCF) was three times higher than that of the control level 60 min after the treatment, indicating H_2O_2 accumulation, and it gradually decreased. Moreover, exposure to 0.3 μM of $HgCl_2$ caused an increase in fluorescence 6 h after the treatment. However, exposure to $HgCl_2$ at concentrations of 3.0 μM or greater did not cause an increase in fluorescence. It is thought that H_2O_2 was also generated at these concentrations, but could not be detected due to inactivation of the cellular esterase necessary to convert DCFH-DA to DCFH, and/or leakage from the cells due to membrane damage caused by $HgCl_2$. The maximum level of fluorescence was approximately two times higher under light conditions than under dark conditions, suggesting that H_2O_2 was derived partly from photosynthesis. These results indicate that accumulation of H_2O_2 is involved inconsequence of the toxicity of mercury.

Keywords: cytotoxicity; H_2O_2; mercury; oxidative stress; Prasinophyceae; Tetraselmis tetrathele

INTRODUCTION

Metal stress is one of the main environmental stresses that affect the production of reactive oxygen species in plants, causing oxidative stress (Ali and Alqurainy 2006). Reactive oxygen species (ROS), such as the superoxide radical $O_2 \cdot^-$, hydrogen peroxide H_2O_2, hydroxyl radical $\cdot OH$, and singlet oxygen $^1O_2$ are highly reactive compared with O2, and may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA damage (Schützendübel and Polle 2002).

There is good evidence that exposure of plants to excessive concentrations of heavy metals results in oxidative injury, such as redox-active metals of Cu and Fe (De Vos et al. 1992, Gallego et al. 1996, Weckx and Clijsters 1996, Knauert and Knauert 2008, Mazhoudi et al. 1997, Yamamoto et al. 1997), and non-redox-active metals such as Cd (Gallego et al. 1996, Lozano- Rodríguez et al. 1997, Chaoui et al. 1997, Piqueras et al. 1999, Romero-Puertas et al. 1999, Schützendübel et al. 2001, Shaw 1995), Zn (Weckx and Clijsters 1997, Prasad et al. 1999, Madhava Rao and Sresty 2000), Ni (Baccouch et al. 1998, Madhava Rao and Sresty 2000), and Hg (Cho and Park 2000, Shaw 1995.).

Recently, much attention has been given to oxidative stress induced by heavy metals in phytoplankton. The studies mainly focus on chlorophycean algae (Gillet et al. 2006, Gorbi et al. 2006, Jamers et al. 2006, Knauert and Knauert 2008, Luis et al. 2006, Mallick 2004, Randhawa et al. 2001, Stoiber et al. 2007, Tripathi et al. 2005, Tripathi and Gaur 2004), with a little research on other groups of phytoplankton, such as *Pavlova viridis* (Prymnesiophyceae) (Li et al. 2006, 2007), *Euglena gracilis* (Euglenophyceae) (Watanabe and Suzuki 2002, 2004) and *Gonyaulax polyedra* (Dinophyceae) (Okamoto et al. 2001). Furthermore, even though mercury is one of most widespread and hazardous organ-specific environmental contaminants, information on its effects on oxidative stress are limited to land plants (Cho and Park 2000, Shaw 1995) and phytoplankton (Okamoto et al. 2001).

Previously, we showed that an abundance of nonprotein thiols was related to tolerance of $HgCl_2$ in the marine phytoplankton *Tetraselmis tetrathele* (Prasinophyceae) (Satoh et al. 1999, 2002). Prasinophyte species are unicellular green algae, and are thought to be an experimentally suitable model organism for the study of marine phytoplankton because they have small genomes, lack cell walls, and

are widely distributed in the ocean (Škaloud et al. 2006, Wawrik et al. 2003). In this report, we examined whether exposure to mercury induced the production of ROS in *T. tetrathele* using a fluorescent dye 2' 7'-dichlorofluorescin diacetate (DCFH-DA). The principle of the analysis is the conversion of the reduced ester, 2', 7'-dichloro-dihydrofuorescin to DCFH by cellular esterase, and further oxidation by active oxygen species to green-fluorescent 2', 7'-dichloro-dihydrofuorescin (DCF) (Royall and Ischiropoulos 1993, Zhu et al. 1994). Moreover, we used a fluorescent dye fluorescein diacetate (FDA), which allows the assessment of the viability of phytoplankton cells including *T. tetrathele* (Satoh et al. 2002, 2003). With reference to the results, we discuss the implications of oxidative stress due to mercury toxicity.

MATERIALS AND METHODS

Plant material
Clonal cultures of *T. tetrathele* were provided by the Tasaki Institute for Marine Biological Research of Tasaki Shinju Co., Ltd. Cells were grown on a light-dark cycle of 12:12 h at 23°C in F medium (Guillard and Ryther 1962) and were incubated under light or dark conditions for at least 3 h prior to the experiment.

Measurement of fluorescence by flow cytometry.
Fluorescent probes DCFH-DA and FDA were purchased from Lambda Probes & Diagnostics (Graz, Australia). They were dissolved in dimethyl-sulfoxide (DMSO) to produce 10 and 12 mM stock, respectively, which were frozen as aliquots at 4°C. The staining of DCFH-DA was examined as described below. For FDA staining, the 12 mM stock solution was diluted to a concentration of 15 µM. Cells were incubated for 60 min in each experiment following the method of Oyama et al. (1994). The fluorescence intensity was measured using a flow cytometer (Cyto-ACE 150, Jasco, Tokyo, Japan). The excitation wavelength was 488 nm, produced by an argon laser, and emission was detected at wavelength of ±530 nm for both fluorechromes. About 3000 cells were analyzed in each experiment.

RESULTS

Initially, we examined the staining of the fluorescent probe DCFH-DA in order to detect the generation of H_2O_2. Figure 1a shows the change over time in average intensities of fluorescence in *T. tetrathele* cells. Fluorescence intensity reached a plateau 20–30 min after application of DCFH-DA at a concentration of 5.0 and 25 µM, and within 10 min after application at a concentration of 100 µM. The higher the concentration of DCFH-DA applied, the higher the maximum levels of fluorescence intensities. Figure 1b shows the changes in fluorescence intensity induced by treatment with $HgCl_2$ stained with 1.0, 5.0, and 25 µM DCFH-DA for 30 min. Staining with 5.0 µM DCFH-DA increased the fluorescence intensity i.e., approximately three times higher than the control level when the cells were treated with 0.4 µM $HgCl_2$, indicating an accumulation of H_2O_2 in the cells. However, staining with 1.0 and 25 µM DCFH-DA did not show a similar increase after treatment with $HgCl_2$ at the same concentration. It is thought that 1.0 µM of DCFH-DA was too low and 25 µM was too high probably due to strong nonspecific fluorescence. Thus, we concluded that the best staining was obtained by treating with 5.0 µM DCFH-DA for 30 min, following which the experiments were performed under these conditions.

Figure 2 shows the changes in fluorescence intensity of DCF after treatment with various concentrations of $HgCl_2$ for 1, 3, and 6 h. Fluorescence intensity increased more than two and three times than that of the control after treatment with 1.0 µM for 1 and 3 h, respectively, indicating that H_2O_2 is generated within 1 h and gradually disappears. An increase in fluorescence intensity was also observed at the 6th h after treatment with 0.3 µM $HgCl_2$. It seems strange that the increase in fluorescence intensity was not observed by treatment with $HgCl_2$ at anytime with concentrations of more than 1.0 µM, rather, intensities of fluorescence were less than the control level in these cases. Probably, H_2O_2 was generated at these concentrations of $HgCl_2$ but could not be detected due to of the inactivation of cellular esterase (as shown in Fig. 4) and/or leakage from the cells due to of membrane damage caused by $HgCl_2$.

Consequently, we examined the time-course change in DCF under light and dark conditions at 1.0 of µM $HgCl_2$ (Fig. 3). Under light conditions fluorescence intensity began to increase 40 min after $HgCl_2$ treatment, reaching a level four times that of the control after 70 min, before beginning to decrease. Whereas, under dark conditions, fluorescence intensity began to increase 30 min after $HgCl_2$ treatment, and reached a maximum of approximately twice the control level after 50 min, before gradually decreasing. Thus, the ratio of increase in fluorescence is about two times higher under light conditions than under dark conditions, and maximum fluorescence was reached slightly later under light conditions. The difference in fluorescence levels between the light and the dark conditions must be partly due to photosynthesis. H_2O_2 is generated as a by-product of oxidative metabolism in chloroplasts (Asada 1996), as well as mitochondria (Levine 1999) and peroxisomes (del Rio et al. 1992).
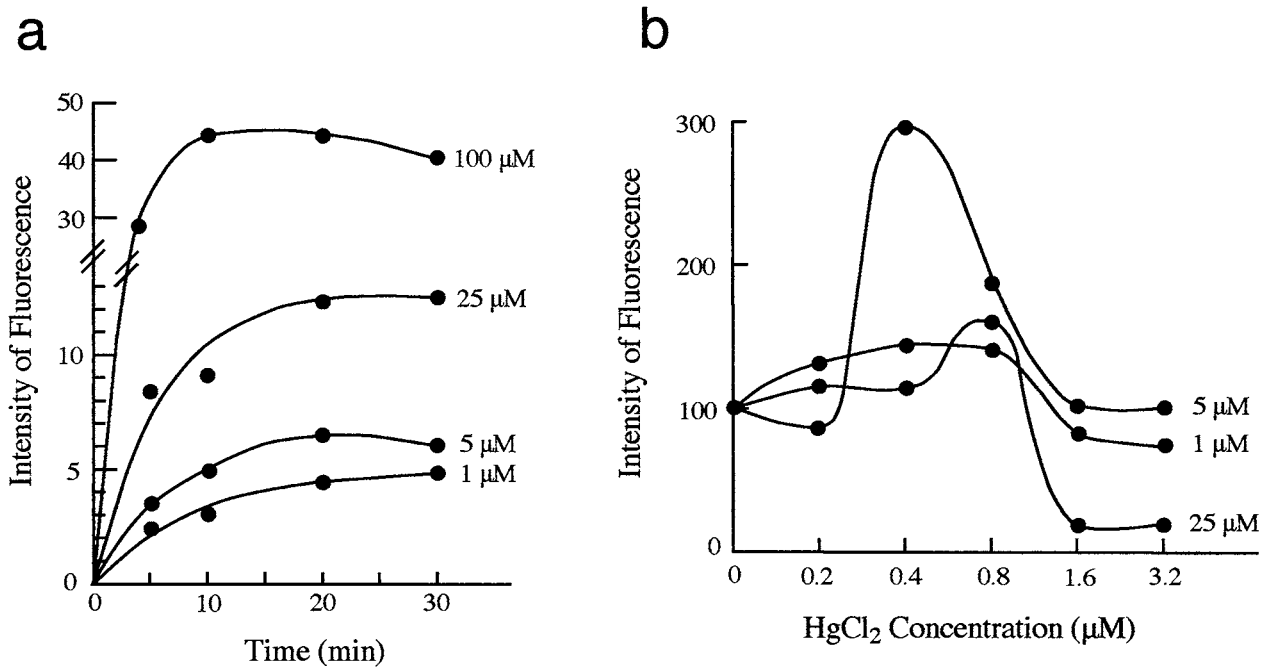
a

b

Fig. 1. Changes in fluorescence intensities of DCF. (a) Changes over time in DCF fluorescence after staining the cells with several concentrations of DCFH-DA as indicated on the panel. Fluorescence intensity is shown as an arbitrary unit. (b) Changes in fluorescence of DCF caused by HgCl2. Cells were exposed to HgCl2 for 1 h at the concentrations indicated on the panel, and fluorescence intensity was measured. DCFH-DA was added 30 min prior to the measurement.
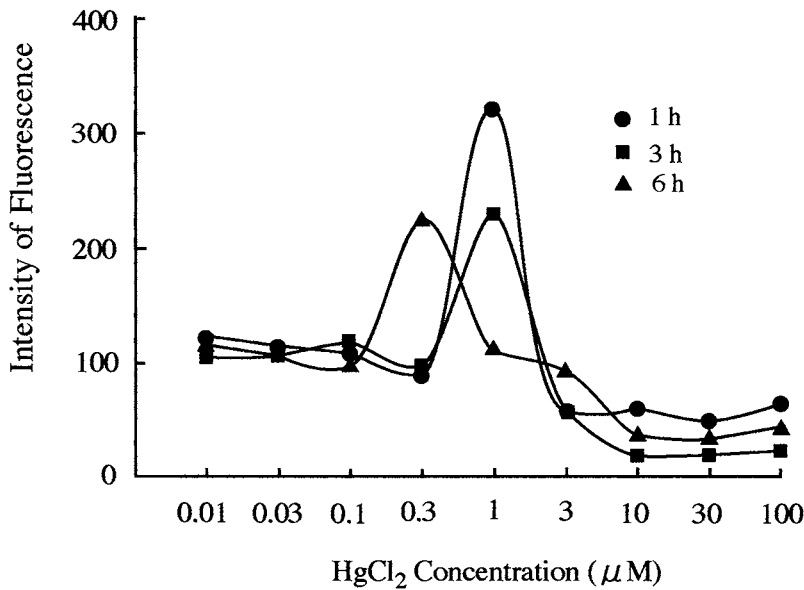

Fig. 2. Changes in fluorescence of DCF caused by HgCl2. Cells were exposed to 1.0 μM HgCl2 for 1, 3, and 6 h, and fluorescence intensity was measured. DCFH-DA was added 30 min prior to the measurement.
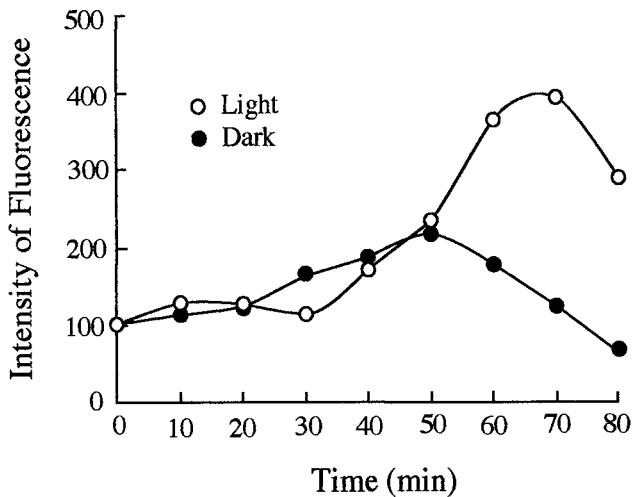
Fig. 3. Change over time in DCF fluorescence under light and dark conditions. Cells were exposed to 1.0 μM HgCl2 and fluorescence was measured at times as indicated. DCFH-DA was added 30 min prior to the measurements.

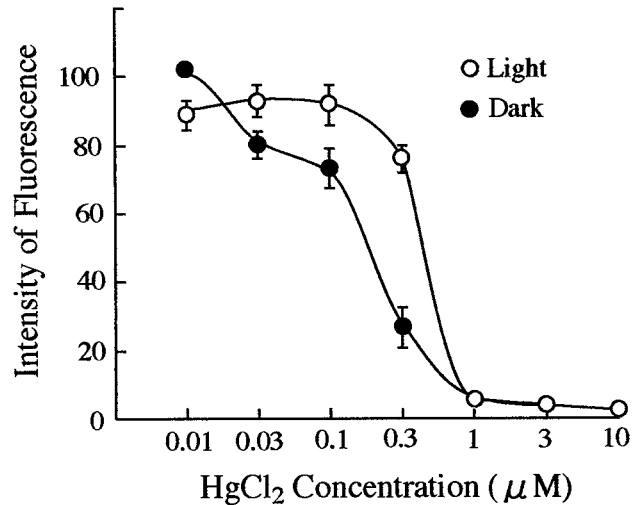**Fig. 4.** Effect of HgCl2 on cell viability under light and dark conditions. Cells were exposed to 1.0 μM HgCl2 and fluorescence was measured at times as indicated. FDA was added 30 min prior to the measurement. Data are presented as means ± standard deviation from a minimum of three independent experiments.

We therefore examined cell viability after treatment with $HgCl_2$ under light and dark conditions by staining with fluorescein diacetate (FDA). Nonfluoresecnt FDA readily enters cells, and is hydrolyzed by intracellular esterase to give fluorescent fluorescein. Figure 4 shows a comparison of fluorescence after $HgCl_2$ treatment. Fluorescence intensity began to decrease at 0.3 and 0.03 μM under light and dark conditions, respectively, indicating that cells were more sensitive to $HgCl_2$ under dark conditions than light conditions. Since the levels of fluorescence intensity are similar in both the light and dark conditions at 1.0 μM, differences in the fluorescence intensity of DCF (Fig. 3) actually reflect the difference in amounts of H_2O_2 generated, although staining with DCFH-DA is also dependent on the activity of esterase as described above. The generation of H_2O_2 does not, therefore, affect the sensitivity of cells to $HgCl_2$ under dark conditions more than under light conditions. Probably, this is due to a difference in the detoxification of mercury by nonprotein thiols, which were more abundant in cells under light conditions than under dark conditions (data not shown).

DISCUSSION

In this study, we showed that the exposure of *T. tetrathele* (Prasinophyceae) cells to $HgCl_2$ caused an accumulation of H_2O_2. Although information on oxidative stress caused by heavy metals in phytoplankton is increasing, it is not well known except for Chrolophycean group of phytoplankton, and oxidative stress caused by mercury is examined only in *Gonyaulax polyedra* (Dinophyceae) (Okamoto et al. 2001). The observation of H_2O_2 accumulation caused by mercury in another phytoplankton group is, therefore, a valuable contribution to the field. Since phytoplankton consists of diverse phylogenetic groups information on the mechanisms of mercury toxicity and elucidation of the antioxidative systems that counteract it in various groups of phytoplankton will be helpful in understanding the effects of mercury pollution in the ocean.

There are two possible sources of the H_2O_2: 1) it was produced *de novo* through a rapid defense system "oxidative burst", and 2) it was generated from normal metabolic processes such as photosynthesis and respiration, and it was not removed due to the damage caused to the detoxification systems by $HgCl_2$. As for the former possibility, it is known that the immune cells of animals or plant cells show a rapid release of ROS, which is known as an "oxidative burst," in response

to pathogenic bacteria, fungi, and viruses (Doke 1997, Lamb and Dixon 1997). However, this kind of mechanism has not been reported in unicellular organisms. As for the latter possibility, Schützendübel and Polle (2002) presented a hypothetical model suggesting that H_2O_2 accumulation in plant cells after Cd exposure is caused by the depletion of the reduced form of glutathione (GSH), inhibition of glutathione reductase (GR) and ascorbate peroxidase (APX). However, we previously showed that exposure of *T. tetrathele* cells to 1.0 μM and less of $HgCl_2$ did not cause depletion of GSH over 24 h (Satoh et al. 1999). Thus, the H_2O_2 accumulation observed in this research may not be due to depletion of GSH. The possibility that $HgCl_2$ inhibits APX remains to be investigated. In addition to GR and APX, there are other antioxidative enzymes, such as superoxide dismutase, catalase, guaiacol peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase (Ali and Alqurainy 2006). Moreover, the effects of $HgCl_2$ on these enzymes remains to be examined.

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