A Polyphenol compound, Rutin suppresses Ultraviolet and Methylviologen-induced Oxidative Cellular Damage on Achlorophyllous Unicellular Eukaryote, *Euglena gracilis* SMZ

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Abstract

Antioxidative effect of the natural antioxidant rutin was examined on the cellular damage induced by ultraviolet light radiation and methyl viologen (MV) in the unicellular flagellate achlorophyllous mutant of *Euglena gracilis* SMZ strain. UV-B irradiation and UV-B and MV combined exposure decreased phospholipid especially phosphatidylethanolamine contents, suggesting that combined exposure of UV and MV induced cell membrane damage under the chronic exposure conditions adapted in the present study. Exposure to UV-B and 200 μM MV induced the highest levels of TBARS, however, the supplementation of rutin suppressed the increase of TBARS.

Key words: achlorophyllous *Euglena gracilis* SMZ, oxidative stress, rutin, methyl viologen, lipid peroxidation, ultraviolet ray irradiation, membrane phospholipid

Introduction

Excess exposure of UV irradiation causes serious damage to all living organisms on the earth [1], and global concerns on UV-induced injury to both aquatic and terrestrial habitants [2, 3] have been growing.

Global environmental pollution, on the other hand, caused by agrochemicals is serious problems to sustain ecosystem healthy. Methyl viologen (MV) (1, 1'-dimethyl- 4, 4'-bipyridinium-dichloride), known as paraquat by commercial name, is a water-soluble herbicide that is most widely used in many areas in the world. The potent herbicidal effect of MV depends on light and oxygen; MV interferes the photo-system by disrupting electron transport and catalyzing the production of active oxygen within the chloroplast [4, 5, 6] to generate superoxide anion ($O_2^{\cdot -}$).

MV is known to cause adverse effect against animal kingdoms, either. It injures fishes, and sometimes is fatal to humans.

E. gracilis is classified as a protist, and cholorophyllous strain has been intensively used as a model for photosynthesis and toxicological studies. While achlorophyllous mutant SMZ strain is unable to survive without supplying organic nutrients. Notably, SMZ also has highly organized subcellular organella equivalent to those of higher mammals so that SMZ can be regarded as a protozoan and an animal model. Therefore, it is interesting to study how SMZ strain would be affected by UV irradiation and herbicide exposure. Not only investigating the adverse effect of UV and herbicide, seeking for prevention measure against the oxidative injury should be important and useful for sustainable ecosystem and for animal health. That is the reason why we examined the effect of pretreatment SMZ strain with rutin.

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Flavonoids are effective reactive oxygen scavengers that protect plants from potential harmful UV-B radiation [7]. Furthermore, rutin, one of flavonoids, has also been reported to be effective in scavenging reactive oxygen species and suppressor of lipid peroxidation [8], and its effect as functional food material to keep human health has been attractive topic in food industries. Previously, the authors reported the effects of UV-A or UV-B irradiation and MV on cell growth, morphology and viability of *E. gracilis* Z and SMZ cells [10]. Combined exposure of UV and MV induced abnormal cell numbers, decrease in the cell viability and suppression of the cell growth in *E. gracilis* Z and SMZ strains whereas pretreatment of rutin suppressed the outbreak of abnormal cells, reduction of growth and viability [10].

In the present study, to assess the effect of UV and MV on the membrane lipid components of *E. gracilis* SMZ cells exposed to UV and MV, quantitative alteration of two major phospholipids (PL), or phospahtidylcholine (PC) and phosphatidylethanolamine (PE), were compared.

Lipid peroxidation levels were also determined in *E. gracilis* SMZ cells after acute exposure to UV and MV, and the effect of rutin supplementation on suppression of lipid peroxidation was assessed by TBARS test with the treated cells.

Materials and Methods

2. 1 Reagents and chemicals

Methyl viologen (1, 1'-dimethyl- 4, 4'-bipyridinium-dichloride), purchased from nacalai tesque® Co., Kyoto, Japan, was dissolved in Koren Hutner (KH) medium and used in the assay at concentration of 200 μM. Control solutions without MV were also prepared. Rutin, purchased from nacalai tesque®, was used to determine whether UV and MV induced cellular damage could be suppressed by supplementation or not. Rutin was dissolved in phosphate buffer solution pH 7.4 to prepare 2 mM solution. All other chemicals used were guaranteed reagent grade.

2. 2 Incubation conditions

E. gracilis SMZ strain were grown at 29℃ (± 1℃) in 5 ml of KH medium [9], in test-tubes under illuminated (3,200 lx) fluorescent light for plant growth on a light／dark cycle of 12／12 hr until early logarithmic growth phase.

2. 3 UV irradiation

UV-A (365 nm, 4 W Black Light Matsushita Electric Co., Osaka) or UV-B (model UVM-57. 302 nm, 6W, equipped with 2 UVG filter to cut UV-C; UVP Inc., Upland, CA, USA), were used for UV irradiation experiment in the present study. UV output was monitored using a radiometer (VLX-3W Vilber lourmat, Torcy, France). *E. gracilis* SMZ cells in the petri-dishes were exposed to UV-A or UV-B by adjusting the distance between the petri-dishes and the UV source, and then the cells were exposed to UV-A or UV-B rays at 3 W m^{-2}, UV-A or UV-B dose at 0.36J・cm^{-2} for each exposures.

2. 4 UV and MV treatments of *Euglena gracilis* SMZ cells for lipid extraction

E. gracilis SMZ cells from a liquid culture, 500 μl of 10^5-10^6 cells／ml were inoculated into 1 liter of KH medium pH 3.5, in Erlenmeyer flasks. The flasks containing cell cultures were placed under the illumination conditions as described above. *E. gracilis* SMZ cell cultures were divided into petri dishes (10^5 cells／ml) and subjected to UV- B irradiation and MV exposure.

MV was added at final concentrations of 200 μM. These petri-dishes were then exposed to UV-A or UV-B as described in section 2.3.

MV concentration was 200 μM, and UV-B was daily exposed at energy levels of 0.36 J cm^{-2} at 305 nm wavelength.

2. 4. 1 Rutin supplementation

The cells were pre-treated with rutin prior to stress loading with UV and/or MV as previously reported [10]. The contents of total lipid and PL of UV and MV exposed *E. gracilis* SMZ cells preliminarily treated with 100 μM rutin were analyzed to examine the suppression of LPO by rutin.

2. 4. 2. Sample preparation for lipid analyses

E. gracilis SMZ cells were harvested immediately after the final exposure of UV irradiation on the third day, including cells exposed to MV single exposure and the non-treated (control) cells, placed in 50 ml glass Pyrex centrifuge tubes, centrifuged at 2,800 × g for 10 min at a temperature of 5 to 10℃. Cell-pellets were washed twice with phosphate saline buffer solution (PBS), pH 7.4. The washed *E. gracilis* SMZ cell-pellets were lyophilized, and subjected to lipid extraction by Bligh and Dyer's method with slight modifications [11].

2. 5. Qualitative and Quantitative Analyses of Phospholipid Fraction by Thin layer chromatography

To identify PL composition, 100 μg of total lipid samples were applied on Silica Gel G TLC plate (20 x 20 cm; 250 μm gel thickness; Merck Co., Darmstadt), and the spots dried under N_2 stream before the TLC plates were developed with chloroform: methanol: water = 65: 25: 4 (v/v/v). After the development, the TLC were dried, spots were located by spraying with 3% copper acetate-8% phosphoric acid and subsequent heating at 180℃ for 20 minutes. PL components were identified and quantified by comparing Rf-values and areas with those of known amount of authentic samples using a TLC scanning densitometer (Shimadzu 9000, Shimadzu, Kyoto, Japan) at 550 nm.

2. 6. Estimation of secondary degradation products of lipid hydroperoxides by TBARS test

To estimate production of secondary degradation products of LPO, TBARS (thiobarbituric acid reactive substance), or malondialdehyde (MDA) values of cells loaded to UV-A or UV-B irradiation with doses of 0.78 J· cm^{-2} at 3 W m^{-2} with or without 200 μM MV and pretreatment with 50 μM and 100 μM rutin were compared [12].

2. 7. Statistical analysis

The data were evaluated by analysis of variance (ANOVA). A value of $p < 0.005$ was considered to be significant.

Results

3. 0. Total lipids

Figure 1 shows total lipid contents of *E. gracilis* SMZ with or without UV and/or MV stress loading. The average total lipid percentage of the dry cell weight basis in the SMZ strain was 15.25 ± 1.5%, however, significant differences were not observed between any experimental conditions.

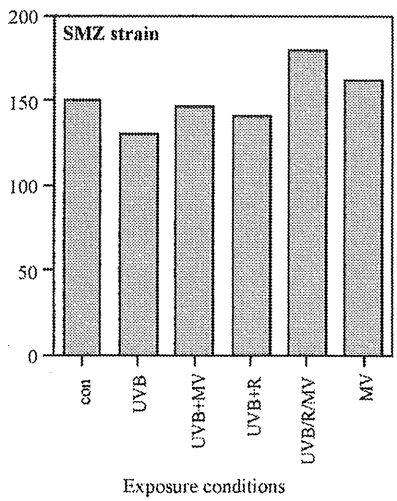

Fig. 1 The total lipid content expressed as mg/g (dry cell) of *E. gracilis* SMZ exposed; without UV and MV (control), to; UV-B irradiation (UVB), UV-B irradiation and 200 μM MV (UVB+MV), UV-B irradiation and 100 uM rutin supplementation (UVB+R), UV-B irradiation and 200 μM MV with 100 μM rutin supplementation (UVB/MV/R), 200 μM MV (MV).

3. 1. Variation of PL compositions

Quantified data of PC and PE are presented as bar graphs in Figure 2 for SMZ strain. The PE content decreased in SMZ cells exposed to UV-B, and UV-B and MV, were PE contents almost 50% less than control ($p > 0.005$) (Fig. 2). The PC content was lower in the *E. gracilis* cells exposed to UV-B single exposure and UV-B and MV treated cells in the SMZ strain (Fig. 2), compared to the control groups.

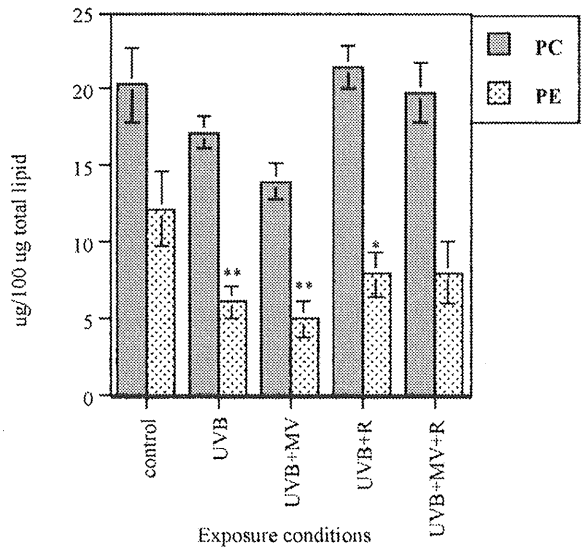

Fig. 2 Phosphatidylethanolamine and phosphatidylcholine contents in *E. gracilis* SMZ exposed to UV and methyl viologen with or without rutin

The PE and PC contents in *E. gracilis* SMZ (Fig. 1) exposed ; without UV and MV (control), to; UV-B irradiation (UVB), UV-B irradiation and 200 μM MV (UVB+200 μM MV), UV-B irradiation and 100 μM rutin supplementation (UVB+R), UV-B irradiation and 200 μM MV with 100 μM rutin supplementation (UVB+MV+R).

3. 2. TBARS level under Exposure to MV and / or UV-A or UV-B and the effect of rutin treatment

Simultaneous exposure to UV-B and MV (UV-B + MV) resulted in the highest levels of TBARS in SMZ strains of *E. gracilis* (Figure 3). UV-B alone and simultaneous exposure of UV-B and MV showed significantly higher TBARS values than control, indicating enhancement of membrane lipid peroxidation.

While cells treatment with 100 μM rutin immediately before the UV-B exposure showed marked decrease in TBARS levels. The 50 μM supplementation also suppressed the TBARS levels to an extent (Fig. 3).

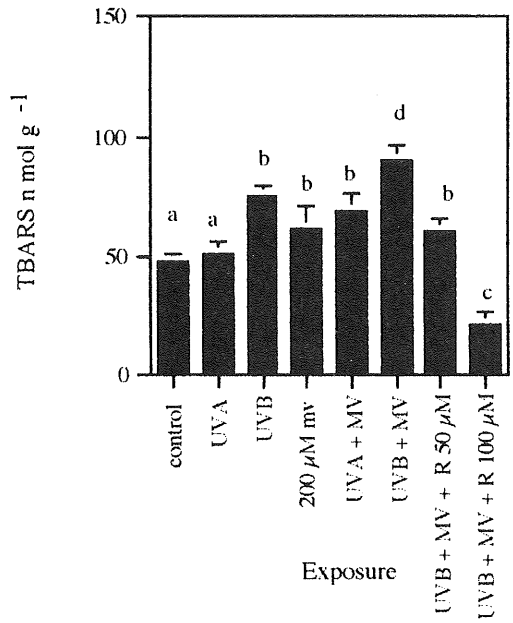

Fig. 3 Levels of TBARS in *E. gracilis* cells exposed to UV and MV and the effect of rutin supplementation

Cells without exposure to UV or MV (Control), UV-A irradiation (UVA), UV-B irradiation (UV-B), 200 μM methyl viologen (200 μ M MV), UV-A irradiation and 200 μ M methyl viologen exposure (UVA+MV), UV-B irradiation and 200 μ M methyl viologen exposure (UV-B+MV), UV-B irradiation and 200 μM methyl viologen exposure with 50 μ M rutin (UV-B+MV+R 50 μM), UV-B irradiation and 200 μM methyl viologen with 100 μ M rutin (UV-B+MV+R100 μ M). The total exposure energy of UV-A and UV-B was 0.72 J $\cdot$ cm^{-2}. Each bar represents the mean value $\pm$ S.D. (n = 9). Cells were analyzed immediately after UV irradiation.

Discussion

Considering that LPO formation is often one of the incidences of excess ultraviolet irradiation, as a parameter for lipid membrane damage, the major phospholipid components of *E. gracilis* under the exposure of UV and MV were assessed in the present study.

UV-B exposure and combined with MV exposed groups contained the lowest PE and PC content in the SMZ strain (Fig. 2). It has generally been accepted that the greater the number of double bonds to PUFA, the more susceptible they are to LPO [16, 17, 18]. It is possible that the decrease in the PC and PE phospholipid components in SMZ strain under UV and MV chronic exposure, are an indication of UV and MV induced membrane damage. Phosphlipids especially PE in *E. gracilis* are abundant in arachidonoic acid (C 20: 4) and eicosapentaenoic acid (C 20: 5), as well as palmitic acid (16: 0) [16]. To specify lipid membrane damage, further analyses of fatty acid components are necessary.

Halliwell and Gutteridge reported previously that secondary products such as flavonols and flavonoids have powerful antioxidant actions, such as scavenging O_2^- and inhibiting lipid peroxidation [17]. Rutin is known to function as an O_2^- scavenger [8, 18], and the levels of TBARS in cells exposed to MV and UV-B were suppressed by the addition of rutin compared with non rutin supplementation in this study. The SMZ strain exposed to UV-B and MV in the presence of 100 μM of rutin showed remarkably low levels of TBARS (Fig. 3).

In order to clarify the mechanism of protecting role of rutin against UV and MV induced oxidative stress, further investigation on the incorporation and intracellular or extracellular behavior of rutin are necessary.

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