Natural Science Research (Tokushima University)
Vol. 30 pp. 5–8 (2017)

Rhamnan sulfate attenuates methylmercury cytotoxicity in rat thymic lymphocytes

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Abstract

Rhamnan sulfate, one of sulfated polysaccharides from seaweeds, is considered to have various characteristics such as antioxidant, anticoagulant, anti-inflammatory, antitumor, contraceptive, and antiviral activities, for the treatment of several diseases. We examined the effect of rhamnan sulfate on thymic lymphocytes treated simultaneously with methylmercury chloride, a toxic organometallic compound, using a flow-cytometric techniques with fluorescent probes, fluo-3-AM (an indicator for intracellular Ca^{2+}) and propidium iodide (an indicator for dead cells). Rhamnan sulfate attenuated the methylmercury-induced increase in cell lethality. This effect of rhamnan sulfate is supposed to be due to the attenuation of methylmercury-induced elevation of intracellular Ca^{2+} levels. Rhamnan sulfate may be useful for the prevention of organometallic intoxication.

Keywords: Rhamnan sulfate; Methylmercury; Cytotoxicity; Intracellular Ca^{2+}; Lymphocytes

1. Introduction

We screened and analyzed 14 edible seaweeds in the ability to adsorb Shiga toxin. The extract of *Ulva linza* Linnaeus (Usuba-aonori) among them well adsorbed Shiga toxin. The absorbing substance in the extract was regarded as a rhamnan sulfate (Badr Badr et al., 2017). Sulfated polysaccharides from seaweeds, including rhamnan sulfate, are considered to possess various biological activities such as antioxidant, anticoagulant, anti-inflammatory, antitumor, contraceptive, and antiviral activities, for the treatment of several diseases (Costa et al., 2010; Wijesekara et al., 2011; Wang et al., 2014). The extracts of some seaweeds, *Halimeda incrassata* (Ellis) Lamouroux and *Bryothamnion triquetrum* (S.G.Gmelim) Howe, exerted protective actions against methylmercury-induced cytotoxicity in mouse hypothalamic neurons (Fallarero et al., 2003). In this study, we examined the effects of rhamnan sulfate isolated from *Ulva linza* Linnaeus on rat thymic lymphocytes treated with methylmercury chloride, using flow-cytometric techniques with appropriate fluorescent probes, to see if rhamnan sulfate possesses a protective action against methylmercury toxicity.

2. Materials and methods

2.1. Chemicals

Rhamnan sulfate was prepared as follows. The extract of the *Ulva linza* Linnaeus seaweed was put on a DEAE-Toyopearl column (2.0 × 20 cm) equilibrated with 20 mM Tris-HCl (pH 7.5). After the column was washed with the same buffer, bound chemical components were eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions that showed the adsorbing ability were combined, concentrated with Amicon ultra-15 (MWCO: 10 kDa), and subjected to the gel filtration with a HiPrep 16/60 Sephacryl S-300 HR column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. Fractions that showed the adsorbing ability were combined, dialyzed against distilled water and freeze-dried. The freeze-dried substance was identified as rhamnan sulfate by GCMS analysis.

Methylmercury chloride was supplied from Tokyo Chemical Company (Tokyo, Japan). Propidium iodide was obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Fluo-

3-AM was purchased from Dojin Chemical Laboratory (Kumamoto, Japan). Other chemicals were obtained from Wako Pure Chemicals unless mentioned.

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments at Tokushima University (TS29-56). The cell suspension was prepared as previously reported (Chikahisa et al., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions (2–4°C). The slices were triturated in chilled Tyrode's solution to dissociate the thymocytes. The cell-containing solution was then passed through a 56-μm diameter mesh to prepare the cell suspension. The cell suspension was incubated at 36–37°C for 1 h before the experiment.

Various concentrations of rhamnan sulfate (10–100 mg in 2 μL water) were added to cell suspensions (2 mL per test tube) and incubated at 36–37°C. A sample from each cell suspension (100 μL) was analyzed by flow cytometry to assess the fungicide-induced changes in cellular parameters. Data acquisition from 2×10^3 cells or 2.5×10^3 cells required 10–15 s.

2.3. Fluorescence measurements of cellular parameters

Cell and membrane parameters were measured using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes. To assess cell lethality, propidium iodide was added to the cell suspension at a final concentration of 5 μM. To estimate changes in intracellular Ca^{2+} levels, Fluo-3-AM was used (Kao et al., 1989). The excitation wavelength for both probes was 488 nM. The emissions were detected at 530 ± 20 nm for Fluo-3 fluorescence and at 600 ± 20 nm for propidium fluorescence. Fluorescence was analyzed by JASCO software (Version 3.06; JASCO, Tokyo, Japan). Fluo-3 fluorescence was monitored in cells that did not exhibit propidium fluorescence because the cells exhibiting propidium fluorescence were supposed to be dead cells. No fluorescence was produced by the reagents used in the study under the present experimental conditions, with the exception of the fluorescent probes.

2.4. Statistical analysis and figure presentation

Statistical analyses were performed with post doc Tukey's multivariate analysis. A P-value less than 0.05 was considered significant. In the results, values (columns and bars in figures) were expressed as the mean and the standard deviation of four samples. Each experiment was repeated three times unless noted otherwise.

3. Results and Discussion

3.1. Changes in cell lethality by rhamnan sulfate, methylmercury, and their combination

The cells were incubated with rhamnan sulfate, methylmercury chloride, or their combination, respectively. The incubation time was 3 h. Interestingly, high concentrations (30–100 μM) of rhamnan sulfate reduced cell lethality under control conditions (Fig. 1). The cell suspension contained intact living cells, damaged living cells, and dead cells. Of damaged living cells, some may undergo dying during the incubation. Therefore, rhamnan sulfate is supposed to ameliorate damaged living cells.

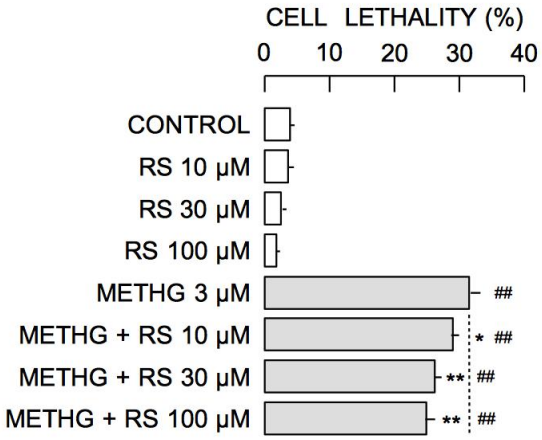

Fig. 1. Changes in cell lethality of cells incubated with rhamnan sulfate (RS), methylmercury (METHG), and their combination. Column and bar show mean and standard deviation of four samples. Symbols (##) indicate significant difference ($P < 0.01$) between control group (CONTROL) and drug-treated groups. Asterisks (*, **) show significant difference ($P < 0.05$, $P < 0.01$) between methylmercury-treated group without and with rhamnan sulfate.

The incubation with 3 μM methylmercury chloride significantly increased the population of

cells exhibiting propidium fluorescence, presumably dead cells. Thus, methylmercury chloride significantly increased cell lethality (Fig. 1). Rhamnan sulfate was applied to the cells at 5 min before the application of methylmercury chloride. Attenuation of methylmercury-induced increase in cell lethality was observed in the case of simultaneous application of 10–100 μM rhamnan sulfate and 3 μM methylmercury chloride (Fig. 1). It may be not surprising because methylmercury induces oxidative stress (Stohs and Bagchi, 1995) and rhamnan sulfate possesses antioxidant activity (Costa et al., 2010; Wijesekara et al., 2011; Wang et al., 2014).

3.2. Rhamnan sulfate attenuates methylmercury-induced augmentation of Fluo-3 fluorescence

Abnormal increase in intracellular Ca^{2+} concentration is one of causes for methylmercury-induced cytotoxicity (Tan et al., 1993; Roos et al., 2012). Oxidative stress increases intracellular Ca^{2+} levels (Ermak and Davies, 2002). Therefore, there was a possibility that rhamnan sulfate attenuated the methylmercury-induced increase in intracellular Ca^{2+} levels. To test the possibility, the fluo-3 fluorescence was compared in the cells treated with rhamnan sulfate, methylmercury chloride, or their combination. The treatment with rhamnan sulfate for 1 h slightly reduced the intensity of fluo-3 fluorescence while methylmercury chloride significantly augmented fluo-3 fluorescence (Fig. 2). Thus, the application of methylmercury chloride greatly increased the intracellular Ca^{2+} concentration.

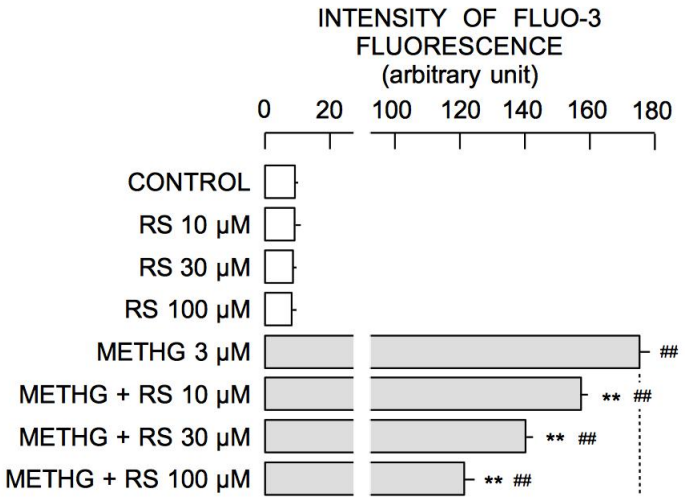

Fig. 2. Changes in intracellular Ca^{2+} levels of cells incubated with rhamnan sulfate (RS), methylmercury (METHG), and their combination. Column and bar show mean and standard deviation of four samples. Symbols (##) indicate significant difference ($P < 0.01$) between control group (CONTROL) and drug-treated groups. Asterisks (**) show significant difference ($P < 0.01$) between methylmercury-treated group without and with rhamnan sulfate.

In the case of simultaneous application of rhamnan sulfate and methylmercury chloride, rhamnan sulfate was added to the cell suspension at 5 min before the application of methylmercury. Thereafter, the cells were incubated with rhamnan sulfate and methylmercury chloride for 1 h before the measurement of flio-3 fluorescence. As shown in Fig. 2, rhamnan sulfate greatly reduced the intensity of fluo-3 fluorescence augmented by methylmercury chloride. Thus, rhamnan sulfate significantly attenuated the methylmercury-induced increase in intracellular Ca^{2+} concentration. It is recognized that toxic cell death is Ca^{2+}-dependent (Schanne et al., 1979). Methylmercury induces oxidative stress, resulting in the elevation of intracellular Ca^{2+} levels that causes cell death (Schanne et al., 1979; Tan et al., 1993; Ermak and Davies, 2002; Roos et al., 2012). Sulfated polysaccharides from seaweeds are considered to possess many biological activities including an

antioxidative activity (Costa et al., 2010; Wijesekara et al., 2011; Wang et al., 2014). The attenuation of methylmercury-induced increase in intracellular Ca^{2+} concentration may be due to the antioxidative action of rhamnan sulfate. In this aspect, further study is necessary.

Conflict of interest

All authors affirm that there are no conflicts of interest to declare.

Acknowledgements

This study was supported by the Tokushima University within the Research Cluster No. 1703021 (Tokushima, Japan) and the Grant-in-Aid for Scientific Research (15K00784) from the Japan Society for the Promotion of Science (Tokyo, Japan).

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Article History:
Received MS: November 21, 2017
Received Revised MS: November 28, 2017
Accepted MS: December 5, 2017