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Zinc increases vulnerability of rat thymic lymphocytes to arachidonic acid under in vitro conditions

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ABSTRACT

Previous studies on the cytotoxicity of arachidonic acid (ARA) elucidated the involvement of oxidative stress and Ca^{2+}. In the present study, the Zn^{2+}-related cytotoxicity of ARA was studied by a flow cytometric technique with appropriate fluorescent probes in rat thymocytes. Addition of 10 μM $ZnCl_2$ enhanced the increase in cell lethality induced by 10 μM ARA. The removal of Zn^{2+} by Zn^{2+} chelators attenuated the ARA-induced increase in cell lethality. Thus, Zn^{2+} is suggested to be involved in ARA cytotoxicity. ARA at 3–10 μM elevated intracellular Zn^{2+} level. The Zn^{2+} chelators attenuated the ARA-induced increase in intracellular Zn^{2+} level while ARA significantly increased intracellular Zn^{2+} level in the presence of 3 μM $ZnCl_2$, suggesting the involvement of external Zn^{2+}. Zn^{2+} reportedly exerts cytotoxic action under oxidative stress induced by hydrogen peroxide, via an excessive increase in intracellular Zn^{2+} levels. Since ARA induces oxidative stress, the simultaneous administration of zinc and ARA may be harmful.

1. Introduction

Polyunsaturated fatty acids are essential fatty acids that humans cannot synthesize and have a variety of nutritional and biochemical properties (Benatti et al., 2004). Various studies have reported the anti-aging actions of polyunsaturated fatty acids (Cole et al., 2010; Janssen and Kiliaan, 2014). However, some omega-3 and 6 polyunsaturated fatty acids exert cytotoxic actions on leukemia cell lines, melanoma cell lines, and lymphocytes in vitro (Finstad et al., 1998; Andrade et al., 2005; Otton and Curi, 2005). Such polyunsaturated fatty acids also kill rat thymic lymphocytes by inducing the release of Ca^{2+} from the endoplasmic reticulum, which causes the release of reactive oxygen species, that leads to cell death, from mitochondria (Prasad et al., 2010). In addition, cell death by polyunsaturated fatty acids is preceded by reduction of both plasma and mitochondrial membrane potential, and occurs via apoptosis of murine thymocytes (Åhs et al., 2011). Oxidative stress and/or excessive elevation in intracellular Ca^{2+} level are involved in the cell death induced by polyunsaturated fatty acids. The relationship between polyunsaturated fatty acids and oxidative stress has been investigated in the studies of Pompéia et al. (2002, 2003).

In our previous study (Matsui et al., 2010), the application of $ZnCl_2$ at low micromolar concentrations exerted cytotoxic action under oxidative stress induced by hydrogen peroxide, in rat thymic lymphocytes. Zn^{2+} is released from cellular thiols under oxidative stress conditions, which then convert thiols to disulfides (Maret, 1994; Jacob et al., 1998; Kinazaki et al., 2011). Thus, the buffering ability that maintains physiological intracellular Zn^{2+} levels appears to decrease in the presence of polyunsaturated fatty acids that induce oxidative stress. Therefore, the cytotoxicity of polyunsaturated fatty acids might be related to Zn^{2+} if polyunsaturated fatty acids induce oxidative stress. To test this hypothesis, the effect of arachidonic acid (ARA), one of polyunsaturated fatty acids, on rat thymic lymphocytes was examined using appropriate fluorescent probes for cell viability and intracellular Zn^{2+} levels, and a flow cytometer to measure fluorescence. And, the cytotoxic action of ARA was further studied. The usage of ARA and zinc as supplements is expected to expand because of their proposed beneficial effects in

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the elderly and infants. Therefore, it is important to further characterize the cytotoxicity of ARA to ensure their safe use.

2. Methods and materials

2.1. Cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). Experimental methods were similar to those described in previous papers. The cell suspension was prepared as previously reported by Chikahisa et al. (1996) and Matsui et al. (2010). In brief, thymus glands dissected from ether-anesthetized rats were sliced under ice-cold conditions. The slices were triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was incubated at 36–37 °C for 1 h before the experiment. It is noted that the cell suspension contained 216.9 ± 14.4 nM zinc derived from cell preparations (Sakanashi et al., 2009).

2.2. Chemicals

ARA, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and linoleic acid (LA) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). FluoZin-3-AM, 5-chloromethylfluorescein diacetate (5-CMF-DA), annexin V-FITC, and propidium iodide were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). The chelators of Zn^{2+}, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DTPA) and N,N,N′,N′-Tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), were purchased from Dojindo (Kumamoto, Japan).

2.3. Fluorescence measurements of cellular parameters

Cell lethality was assessed using propidium iodide, the dye was added to the cell suspension at a final concentration of 5 μM. Exposure of phosphatidylserine on outer surface of cell membranes, a phenomenon during apoptosis, was detected using annexin V-FITC. FluoZin-3-AM (500 nM) was added to the cell suspension to assess the change in intracellular Zn^{2+} concentrations (Gee et al., 2002). FluoZin-3 fluorescence was measured from the cells that were not stained with propidium (living cells with intact membranes). The cellular content of nonprotein thiols was estimated with 5-CMF-DA. The correlation coefficient between the intensity of 5-CMF fluorescence monitored from rat thymocytes and the cellular content of glutathione was 0.965 (Chikahisa et al., 1996). Fluorescence intensity was measured and analyzed using a flow cytometer (CytoACE-150, JASCO, Tokyo, Japan). The excitation wavelength was 488 nm. Fluorescence of FITC, FluoZin-3, and 5-CMF was detected at 530 ± 20 nm. Propidium fluorescence was detected at 600 ± 20 nm.

2.4. Statistical analysis

Statistical analyses were performed using an ANOVA with a post-doc Tukey's multivariate analysis. P-values of less than 0.05 were considered significant. The results (including columns and bars in figures) were expressed as the mean and standard deviation of the 4–42 samples.

3. Results

3.1. Changes in cell lethality by ARA, DHA, EPA, and LA

As shown in Fig. 1A, the incubation of rat thymocytes with 10 μM ARA for 1 h increased the population of cells exhibiting propidium fluorescence (the population of dead cells). The increase in cell lethality by 10 and 30 μM ARA was statistically significant. This was not the case for 3 μM ARA (Fig. 1B). DHA at a concentration of 30 μM, but not 10 μM, significantly increased the lethality (Fig. 1B). EPA and LA did not significantly affect the lethality at concentrations of up to 30 μM (Fig. 1B).

In the experiments described below, the concentration of ARA to

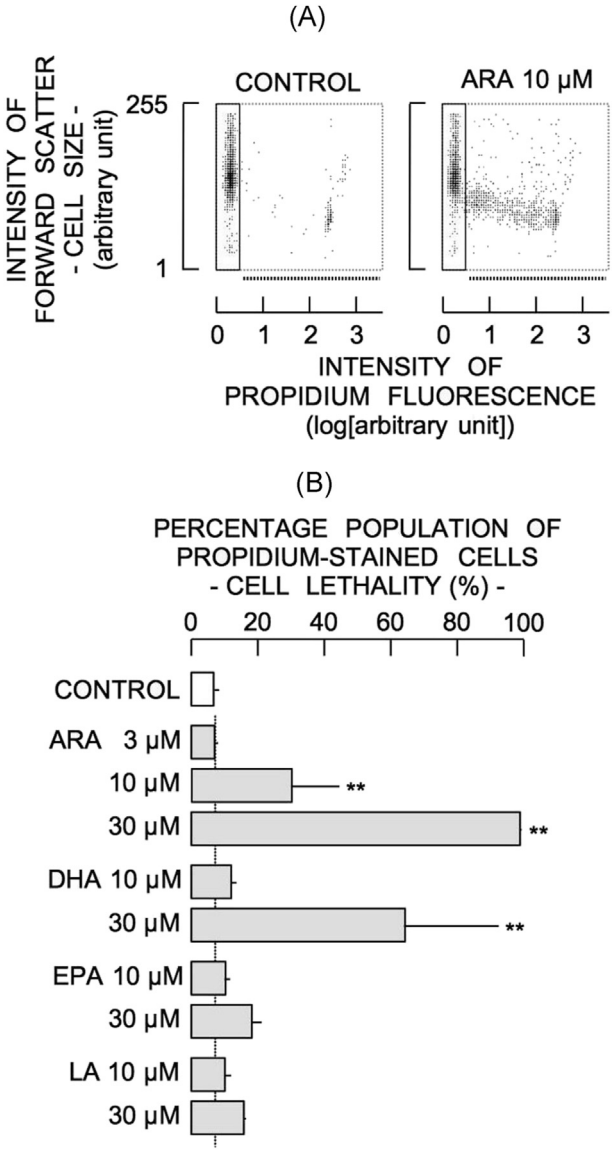

Fig. 1. Changes in the population of cells exhibiting propidium fluorescence after treatment with unsaturated fatty acids. (A) Cytogram of ARA-induced change (ordinate: forward scatter, abscissa: propidium fluorescence). Effect was examined 1 h after the start of the application of 10 μM ARA. Each cytogram was constructed with 2500 cells. Dotted line indicates the population of cells exhibiting propidium fluorescence. (B) Concentration-dependent changes in the population of cells exhibiting propidium fluorescence after treatment with unsaturated fatty acids. Column and bar respectively indicate the mean population and standard deviation of the 8–42 samples. Asterisks (**) show significant change ($P < 0.01$) between control group (CONTROL) and test group.