

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 $10\ \mu\text{M}$ ZnCl_2 enhanced the increase in cell lethality induced by $10\ \mu\text{M}$ 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 $3\text{--}10\ \mu\text{M}$ 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 $3\ \mu\text{M}$ 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. (155 words)

Keywords: arachidonic acid; cytotoxicity; lymphocyte; oxidative stress; zinc

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

Methods and Materials

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).

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).

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 μ M. 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.

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.

Results

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 decrease cell viability was selected to characterize the cytotoxic action. ARA at 10 μM started to decrease cell viability of rat thymocytes (Fig. 1). The plasma concentration of arachidonic acid in human was reported to be 1143 ± 468 ng/mL (Shinde et al., 2012). Calculated molar concentration is 3.7 ± 1.5 μM . Therefore, the test concentration of ARA (10 μM) is higher than plasma concentration of ARA in healthy human.

(Figure 1 near here)

ARA-induced increase in population of dead cells positive to annexin V

To see if the apoptotic process is involved in the cytotoxicity of ARA, the experiments were performed using annexin V-FITC. The treatment with 10 μM ARA for 1 h increased the population of dead cells positive to annexin V, one of markers for apoptosis, from 2.2 ± 0.2 % (mean \pm SD of 4 samples) to 27.8 ± 1.5 %. Furthermore, the cell size, estimated from the change in forward scatter, of annexin V-positive dead cells was 97.0 ± 6.5 (mean \pm SD in arbitrary unit of 4 samples) while it was 167.6 ± 1.0 in the case of intact living cells. There were no significant changes in the population of annexin V-positive living cells and annexin V-negative dead cells by the 1 h treatment with 10 μM ARA. The changes in some parameters suggest apoptotic events in the ARA-induced cytotoxicity.

Zinc-induced increase in cytotoxicity of ARA

There was a possibility that the simultaneous application of micromolar ZnCl_2 further

increased the cytotoxicity of ARA because ARA exhibited oxidative stress (Pompéia et al., 2002, 2003) and ZnCl₂ induced cell death under oxidative stress (Matsui et al., 2010). As shown in Fig. 2A, the simultaneous application of 3–10 μM ZnCl₂ and 10 μM ARA further increased the population of cells exhibiting propidium fluorescence. The increase in cell lethality by the addition of ZnCl₂ was statistically significant in the case of 10 μM ZnCl₂. To deduce if Zn²⁺ is involved in the ARA-induced increase in cell lethality, the effects of Zn²⁺ chelators, DTPA and TPEN, were tested. Both Zn²⁺ chelators significantly reduced the ARA-induced increase in cell lethality (Fig. 2B).

(Figure 2 near here)

Effects of celecoxib and indomethacin on ARA-induced increase in cell lethality

ARA is metabolized to several types of bioactive substances such as prostaglandins by cyclooxygenases (Smith et al., 1996). To determine if the metabolites of ARA are involved in the induction of cell death, the effect of 10 μM ARA was examined in the presence of cyclooxygenase inhibitors such as indomethacin and celecoxib (Rao and Knaus, 2008). Respective cyclooxygenase inhibitors were applied to the cells just before the start of ARA application. As shown in Fig. 3, 1 μM indomethacin and 1 μM celecoxib partly, but significantly, attenuated the ARA-induced increase in cell lethality.

(Figure 3 near here)

Increase in intracellular Zn²⁺ levels by ARA

Incubation with 10 μM ARA for 1 h shifted the histogram of FluoZin-3 fluorescence of rat thymocytes to a direction of higher intensity (Fig. 4A), indicating the ARA-induced elevation of intracellular Zn²⁺ levels. The mean intensity of FluoZin-3 fluorescence in the presence of 10 μM ARA was 74.2 ± 8.8 (mean ± SD of 8 samples in arbitrary unit) while it was 21.2 ± 1.9 under control conditions. Effects of 1–10 μM ARA were summarized in Fig. 4B. Significant augmentation of FluoZin-3 fluorescence was observed in the cases of 3–10 μM ARA (Fig. 4B).

(Figure 4 near here)

ARA-induced changes of intracellular Zn²⁺ levels in the presence of Zn²⁺ chelators and ZnCl₂

The removal of external Zn²⁺ by 10 μM DTPA, a chelator of external Zn²⁺, reduced the control level of FluoZin-3 fluorescence (Fig. 5A). Even in the presence of DTPA, 10 μM ARA significantly increased the intensity of FluoZin-3 fluorescence from 10.0 ± 2.7 (control mean value ± SD of 10 samples in arbitrary unit) to 29.3 ± 5.1 (Fig. 5A). ARA at 10 μM increased the fluorescence intensity, in the absence of DTPA, from 13.2 ± 2.4 to 50.6 ± 5.2. Thus, the

removal of external Zn^{2+} by DTPA attenuated the ARA-induced augmentation of FluoZin-3 fluorescence intensity. TPEN, a chelator of intracellular Zn^{2+} , at 10 μ M completely diminished the FluoZin-3 fluorescence (Fig. 5A). The addition of 3 μ M $ZnCl_2$ elevated the control level of FluoZin-3 fluorescence (Fig. 5B). The application of ARA increased the mean FluoZin-3 fluorescence intensity from 51.1 ± 12.3 to 964.1 ± 523.1 in the presence of 3 μ M $ZnCl_2$ (Fig. 5B).

(Figure 5 near here)

Effect of ARA on cellular content of nonprotein thiols

ARA increased the intensity of FluoZin-3 fluorescence in the presence of DTPA (Fig. 5A), indicating the intracellular release of Zn^{2+} . Intracellular Zn^{2+} forms a complex with the thiol group of proteins and nonproteins (Jacob et al., 1998). Modification from a thiol to disulfide, by oxidative stress, releases Zn^{2+} from the proteins and nonproteins (Maret, 1994). Therefore, to determine if ARA reduces the cellular content of nonprotein thiols, the change in the intensity of 5-CMF fluorescence by ARA was examined. Incubation with 3 μ M ARA for 1 h significantly increased the intensity of 5-CMF fluorescence from 90.1 ± 9.3 (control level of 8 samples in arbitrary unit) to 127.4 ± 6.5 , while a significant decrease to 61.9 ± 13.9 was observed in the case of 10 μ M ARA (Fig. 6). The application of 10 μ M TPEN decreased the intensity of 5-CMF fluorescence under control conditions. In the presence of TPEN, 3 μ M ARA slightly increased the intensity of 5-CMF fluorescence from 81.2 ± 4.9 (control level in arbitrary unit) to 90.1 ± 13.1 (Fig. 6). This change was not statistically significant. The significant decrease by 10 μ M ARA was also observed in the presence of TPEN. Thus, the small increase in intracellular Zn^{2+} level by 3 μ M ARA is supposed to increase cellular content of nonprotein thiols as described in the case of $ZnCl_2$ (Kinazaki et al., 2011).

(Figure 6 near here)

Discussion

Cytotoxic actions of ARA have been reported in the papers by Pompéia et al. (2002, 2003), Prasad et al. (2010), and Åhs et al. (2011), suggesting the involvement of oxidative stress and intracellular Ca^{2+} . In the present study, the Zn^{2+} -related cytotoxicity of ARA was revealed as follows. The addition of 10 μ M $ZnCl_2$ to the cell suspension increased cell lethality induced by 10 μ M ARA, and the removal of Zn^{2+} by the Zn^{2+} chelators, 10 μ M DTPA and 10 μ M TPEN, attenuated the ARA-induced increase in cell lethality (Fig. 2). Thus, Zn^{2+} is supposed to be

partly involved in ARA cytotoxicity. ARA at 3–10 μM augmented the intensity of FluoZin-3 fluorescence in a concentration-dependent manner, indicated by the ARA-induced increase of intracellular Zn^{2+} levels (Fig. 4). Zn^{2+} chelators attenuated the ARA-induced augmentation of FluoZin-3 fluorescence while ARA significantly increased the intensity of FluoZin-3 fluorescence in the presence of 3 μM ZnCl_2 (Fig. 5), suggesting the involvement of external Zn^{2+} . Zn^{2+} is necessary for the development and function of lymphocytes (Shankar and Prasad, 1998; Haase and Rink, 2014). Therefore, the excessive increase in intracellular Zn^{2+} levels by ARA may disturb cellular physiological functions that are related to Zn^{2+} . Furthermore, in rat thymic lymphocytes, Zn^{2+} exerts cytotoxic action under oxidative stress induced by hydrogen peroxide (Matsui et al., 2010). Previous studies on ARA cytotoxicity reveal the involvement of oxidative stress (Pompéia et al., 2003; Prasad et al., 2010; Åhs et al., 2011). If ARA induces oxidative stress, the cellular content of nonprotein thiols would decrease. Nonprotein thiols such as metallothioneins are required to maintain the intracellular homeostasis of Zn^{2+} (Thirumoorthy et al., 2011). Therefore, a reduced ability to maintain Zn^{2+} homeostasis and increased influx of Zn^{2+} can induce an excessive increase in intracellular Zn^{2+} levels. Cellular Zn^{2+} dyshomeostasis is linked to a number of human pathologies (Rink and Gabriel, 2000; Prasad, 2013). In the present study, thymocytes were used as an experimental model. They are premature T lymphocytes derived from bone marrow hematopoietic progenitor cells that reach the thymus via circulation. A disrupted zinc homeostasis affects lymphocytes, leading to impaired formation, activation, and maturation (Maares and Haase, 2016). Thus, the simultaneous and continuous administration of zinc and ARA may disturb thymus-dependent T-lymphocyte-mediated immune responses.

Supplementary zinc is recommended in many clinical cases including diseases attributed to aging (Prasad, 2013; Roohani et al., 2013). ARA is thought to prevent and/or improve age-related decline in brain and cardiovascular system function (Kiso, 2011; Bell et al., 2014). Therefore, both zinc and ARA are occasionally used as a supplement by elderly persons. As shown in Fig. 5, ARA significantly increased intracellular Zn^{2+} levels in the presence of ZnCl_2 . If one takes zinc and ARA simultaneously, an unpredicted increase in intracellular Zn^{2+} levels might occur, resulting in harmful physical or mental effects.

This may be also the case in infants. It was reported that in very preterm infants, feeding of ARA-supplemented human milk in the early neonatal period, was associated with better recognition, memory, and higher problem-solving scores at 6 months (Henriksen et al., 2008). Supplementation with zinc maintained normative developmental trajectories, for selected

measures of attention, during the first 18 months of life (Colombo et al., 2014). Therefore, the possibility that some infants simultaneously take zinc and ARA cannot be ruled out.

The plasma concentration of ARA in humans was reported to be 1143 ± 468 ng/mL (Shinde et al., 2012). Calculated molar concentration is 3.7 ± 1.5 μ M. Sánchez et al. (2009) reported the relationship between zinc intake and plasma zinc concentration in adult humans. In the mean zinc intake of 12.2 ± 7.1 mg/day for males and 9.0 ± 4.4 mg/day for females, mean plasma zinc concentrations were 17.4 ± 6.6 μ M and 16.3 ± 6.2 μ M, respectively. Therefore, the concentrations of ARA and ZnCl₂ in this in vitro study may be plausible. However, it is noted that the plasma zinc concentration is not equal to Zn²⁺ concentration because Zn²⁺ is bound to thiols of proteins and nonproteins under the in vivo conditions. The caution is required to extrapolate this in vitro results to humans.

Acknowledgments

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Conflict of interests

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

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Figure legends

Figure 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 μ M 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.

Figure 2. Effects of $ZnCl_2$ (A) and Zn^{2+} chelators (B) on the ARA-induced change in the population of cells exhibiting propidium fluorescence. Column and bar respectively indicate mean population and standard deviation of the 8–42 samples. Asterisks (**) show significant change ($P < 0.01$) between control group (CONTROL) and test group. Symbols (##) indicate significant changes ($P < 0.01$) between the groups indicated with arrows.

Figure 3. Effects of cyclooxygenase inhibitors on ARA-induced change in the population of cells exhibiting propidium fluorescence. 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. Symbols (##) indicate significant changes ($P < 0.01$) between the groups indicated with arrows.

Figure 4. Change in intensity of FluoZin-3 fluorescence by ARA. (A) ARA-induced shift of the FluoZin-3 fluorescence histogram. Each histogram was constructed with 2500 cells. (B) Concentration-dependent change in the intensity of FluoZin-3 fluorescence by ARA. Column and bar respectively indicate the mean intensity and standard deviation of 8 samples. Asterisks (**) show significant change ($P < 0.01$) between control group (CONTROL) and test group.

Figure 5. Effects of Zn^{2+} chelators (A) and $ZnCl_2$ (B) on ARA-induced change in the intensity of FluoZin-3 fluorescence. Column and bar respectively indicate the mean intensity and standard deviation of 8 samples. Asterisks (**) show significant change ($P < 0.01$) between

control group (CONTROL) and test group. Symbols (##) indicate significant changes ($P < 0.01$) between the groups indicated with arrows.

Figure 6. Changes in intensity of 5-CMF fluorescence by ARA in the absence and presence of TPEN. Column and bar respectively indicate the mean intensity and standard deviation of 8 samples. Asterisks (**) show significant change ($P < 0.01$) between control group (CONTROL) and test group. Symbols (##) indicate significant changes ($P < 0.01$) between the groups indicated with arrows.

Figure 1

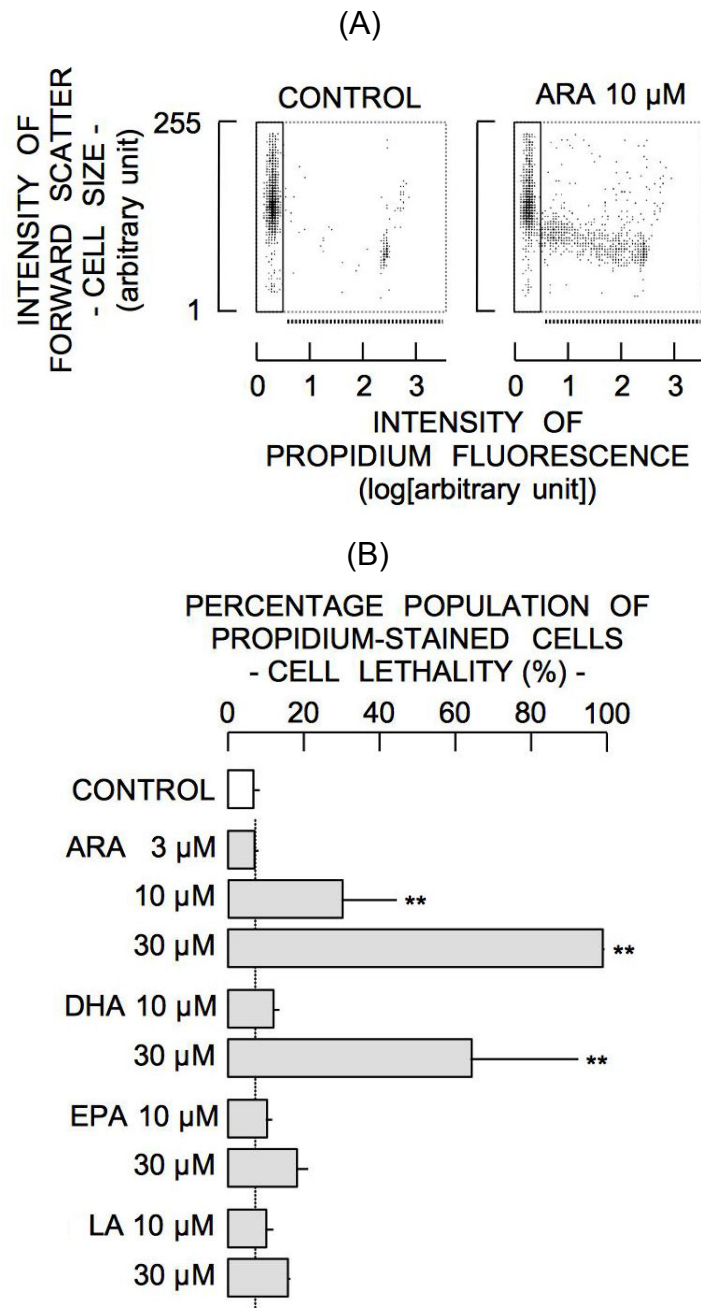


Figure 2

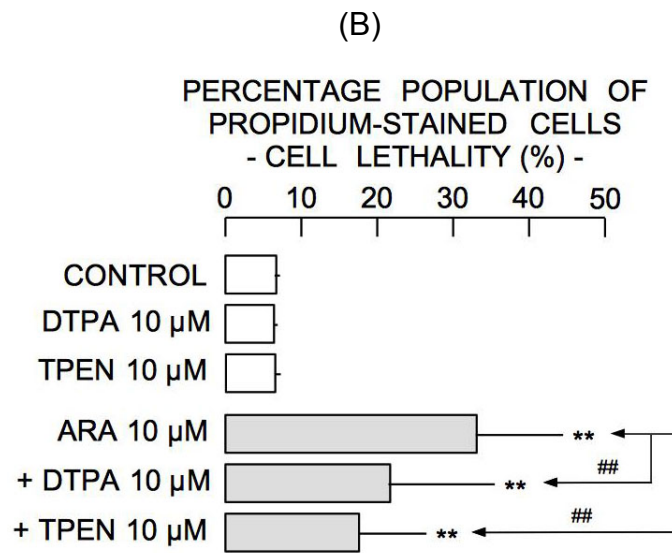
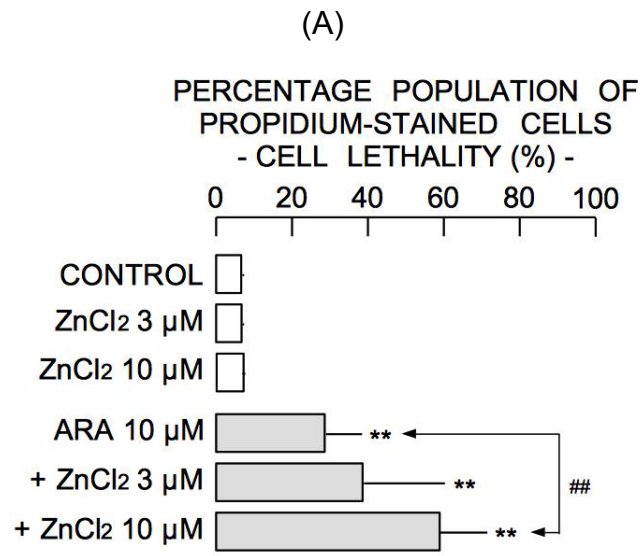


Figure 3

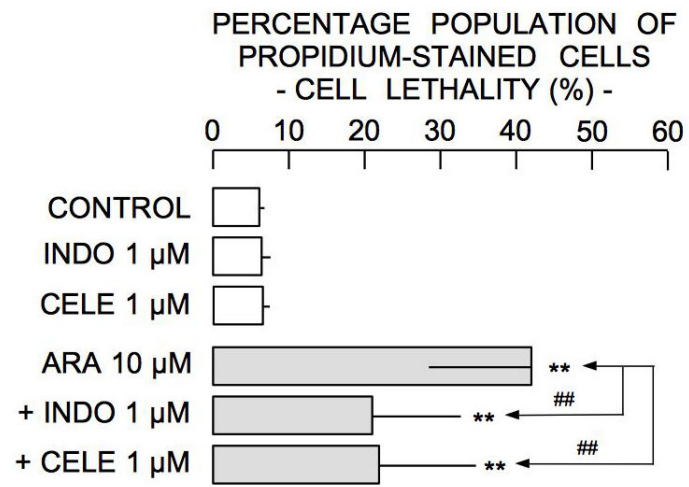


Figure 4

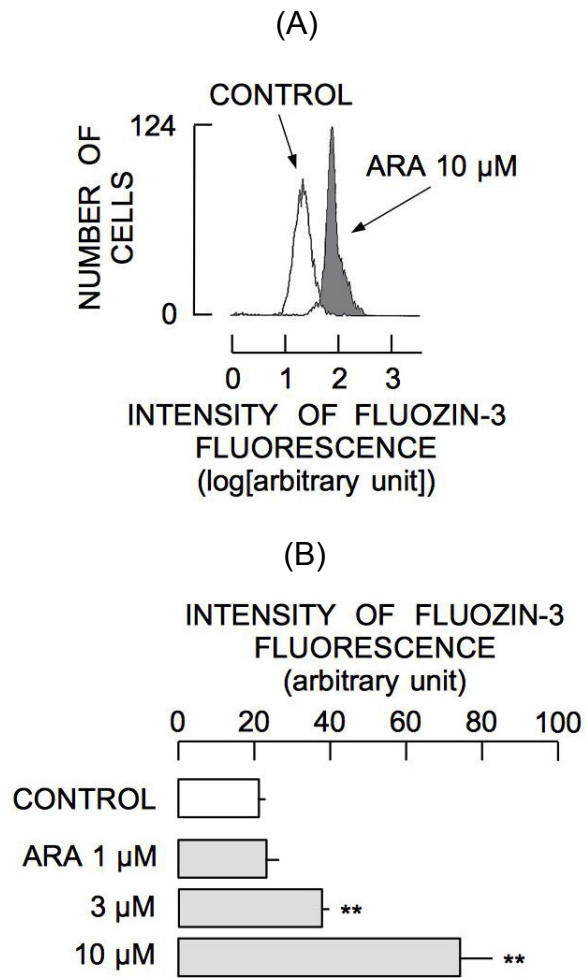


Figure 5

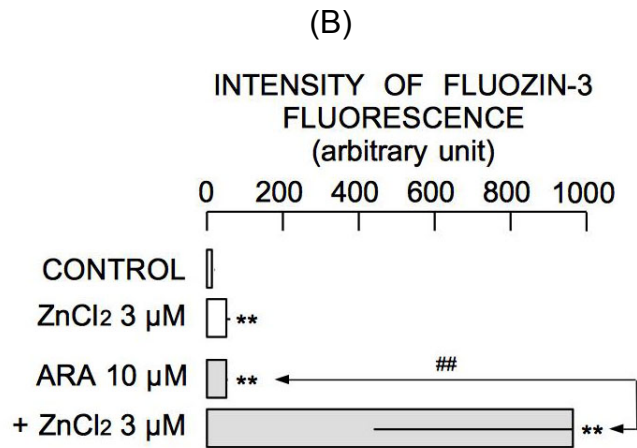
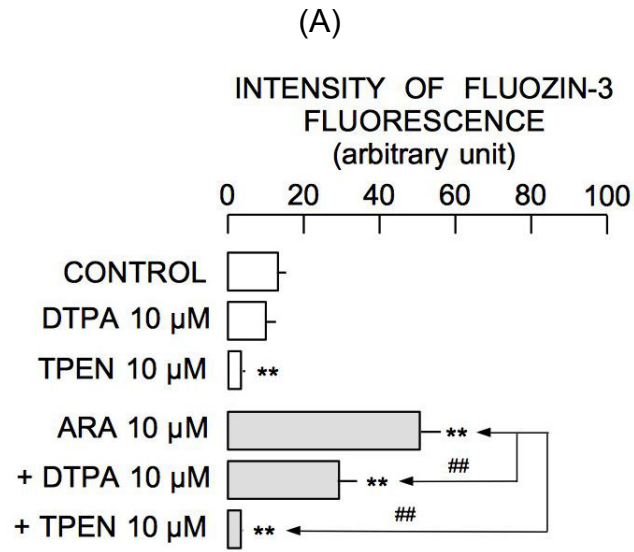


Figure 6

