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Dithiocarbamate fungicides increase intracellular Zn^{2+} levels by increasing influx of Zn^{2+} in rat thymic lymphocytes

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ABSTRACT

Dithiocarbamate fungicides are used as alternative antifouling agents to highly toxic organotin antifouling agents, such as tri-*n*-butyltin and triphenyltin. There are some concerns regarding their environmental and health risks. It has been shown that tri-*n*-butyltin increases intracellular Zn^{2+} levels of mammalian lymphocytes. Therefore, we examined the effects of dithiocarbamate fungicides (Ziram, Thiram, and Zineb) on rat thymic lymphocytes using a flow-cytometric technique to elucidate how these fungicides affect intracellular Zn^{2+} levels. We further determined whether the agents increase intracellular Zn^{2+} and/or Ca^{2+}, because both Zn^{2+} and Ca^{2+} are intracellular signals in lymphocytes, and excessive increases in their intracellular concentrations can have adverse effects. Dithiocarbamate fungicides increased intracellular Zn^{2+} levels, without affecting intracellular Ca^{2+} levels. Ziram was the most potent compound, increasing intracellular Zn^{2+} levels *via* Zn^{2+} influx. Ziram (1 μM) greatly decreased the cellular nonprotein thiol content, and Zn^{2+} chelators attenuated the Ziram-induced decrease. Ziram increased the population of annexin V-positive cells in a Zn^{2+}-dependent manner. Therefore, we propose that dithiocarbamate fungicides induce Zn^{2+} influx, resulting in an excessive elevation of intracellular Zn^{2+} levels, leading to the induction of apoptosis. This study gives a basic insight into the mechanisms of dithiocarbamate fungicide-induced adverse events.

1. Introduction

In agriculture, dithiocarbamate fungicides, such as Ziram and Thiram, are used to protect various fruits and vegetables from fungal infection [1,31,44]. Furthermore, dithiocarbamate fungicides are employed as antifouling agents, because the use of highly toxic organotins, such as tri-*n*-butyltin and triphenyltin, was banned [12,47]. There are increasing concerns regarding the environmental and health risks of dithiocarbamate biocide use [2–4,32,44]. Several papers have evaluated the cellular toxicity of dithiocarbamate fungicides. For example, these fungicides can induce oxidative stress [5,13,19,34]. The cellular thiol content was also reduced in preparations treated with these fungicides [6,18]. The modification of thiols to disulfides releases Zn^{2+} from protein and nonprotein sources [33]. Thus, fungicides may cause a secondary increase in intracellular Zn^{2+} levels. Furthermore, these fungicides could also directly induce Zn^{2+} influx into cells, resulting in an increase in intracellular Zn^{2+} levels. This excessive increase in intracellular Zn^{2+} could also cause oxidative stress, leading to the reduction of cellular thiol content [38,41,46].

Zn^{2+} is an intracellular messengers in lymphocytes [11,22,20]. Therefore, it is important to elucidate how dithiocarbamate fungicides affect intracellular Zn^{2+} levels. In addition, it is necessary to determine whether the fungicides enhance Zn^{2+}, Ca^{2+}, or both, because Zn^{2+} augments the fluorescence of some Ca^{2+} indicators, resulting in misleading observations regarding intracellular Ca^{2+} levels. In this study, we examined the effects of Ziram, Thiram, and Zineb on rat thymic lymphocytes using a flow-cytometric technique with appropriate fluorescent probes. The study may provide cellular data regarding the mechanism of dithiocarbamate fungicide-induced adverse effects.

2. Materials and methods

2.1. Chemicals

Ziram and Thiram were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Zineb was obtained from Wako

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Pure Chemicals (Osaka, Japan). The purity of Ziram, Thiram, and Zineb was 99.9%, 100%, and 96.7%, respectively. Propidium iodide, FluoZin-3-*tetra*(acetoxymethyl)ester (FluoZin-3-AM), and 5-chloromethylfluorescein diacetate (5-CMF-DA) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Fluo-3-AM and the Zn^{2+} chelators, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and *N*,*N*,*N*′,*N*′-*tetrakis*(2-pyridylmethyl)-ethylenediamine (TPEN), were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). A23187, an ionophore for divalent metal cations, was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). 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 the University of Tokushima (No. 05279).

The cell suspension was prepared as previously reported [8,35]. 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. Importantly, the zinc concentration in Tyrode's solution was 32.4 ± 4.0 nM in the case of Tyrode's solution [40]. This significant increase in zinc concentration was probably due to the reagents, probably containing very trace zinc, that were used to prepare the solution. Furthermore, the zinc concentration in the solution that was obtained after removing the cells from cell suspension by a filtration (a pore diameter: 0.22 μM) was 216.9 ± 14.4 nM [40]. Thus, it is likely that the cell suspension contains trace zinc derived from the cell preparation.

Various concentrations of dithiocarbamate fungicides (0.03–3 mM fungicide in 2 μL DMSO) were added to cell suspensions (2 mL per test tube) and incubated at 36–37 °C for 1–3 h. 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. The excitation wavelength for the fluorescent probes used in this study was 488 nM. The emissions were detected at 530 ± 20 nm for FluoZin-3, Fluo-3, and 5-CMF, and at 600 ± 20 nm for propidium iodide. Fluorescence was analyzed by JASCO software (Version 3.06; JASCO, Tokyo, Japan). FluoZin-3, Fluo-3, and 5-CMF fluorescence were 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.

To assess cell viability using propidium iodide, the dye was added to the cell suspension at a final concentration of 5 μM. Since propidium stains dead cells and/or cells with compromised membranes, the measurement of propidium fluorescence in cells can be used to assess viability. To estimate the change in intracellular Zn^{2+} levels, FluoZin-3-AM was used [16,35]. The cells were incubated with 500 nM FluoZin-3-AM for 60 min at least before the fluorescence measurement and drug application. To estimate changes in intracellular Ca^{2+} levels, Fluo-3-AM was used [7,27]. The cells were incubated with 500 nM Fluo-3-AM for 60 min at least before the fluorescence measurement and drug application. 5-CMF-DA was used to monitor changes in the cellular non-protein thiol content, presumably glutathione [8]. The cells were incubated with 1 μM 5-CMF-DA for 30 min before the fluorescence measurements. It is noted that 5-CMF-DA was applied to the cells 30 min after the start of Ziram application. Therefore, the intensity of 5-CMF fluorescence reflects the drug-induced change of cellular glutathione content. The relationship between the intensity of 5-CMF fluorescence monitored from rat thymocytes and the cellular content of glutathione and the correlation coefficient between them was 0.965 in rat thymocytes [8]. Therefore, it is possible to estimate the change in cellular content of glutathione by the use of 5-CMF fluorescence.

Exposure of phosphatidylserine on outer surface of cell membranes, a phenomenon during early stage of apoptosis, was detected using annexin V-FITC [29]. The cells were incubated with annexin V-FITC (10 μL/mL) for 30 min before the measurement. Thus, the cells were initially incubated with Ziram for 30 min and then annexin V-FITC was added to the cell suspension in the continued presence of Ziram.

2.4. Statistical analysis and figure presentation

Statistical analyses were performed by ANOVA, 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

3.1. Changes in cell lethality and FluoZin-3 fluorescence by dithiocarbamate fungicides

Incubation of cells with 0.3–1 μM Ziram, 0.3–1 μM Thiram, or 1–3 μM Zineb for 3 h did not induce cell death in rat thymocytes. The concentrations of dithiocarbamate fungicides used in this study did not increase the population of dead cells.

As shown in Fig. 1A, the incubation of cells with 0.3–1 μM Ziram, 0.3–1 μM Thiram, and 3 μM Zineb shifted the histogram of FluoZin-3 fluorescence to a direction of higher intensity, indicating the drug-induced increased intracellular Zn^{2+} levels. A rapid increase in the intensity of FluoZin-3 fluorescence was observed after the application of Ziram and Thiram. The fluorescence attained a peak and steady state within 30 min of application. Therefore, the effects of dithiocarbamate fungicides on the fluorescence were examined 30 min after application. The potency of dithiocarbamate fungicides varied from agent to agent. Ziram (0.03 μM) initially increased the intensity of FluoZin-3 fluorescence, and further increases in Ziram concentration (0.1–1 μM) exhibited further augmentation of FluoZin-3 fluorescence in a concentration-dependent manner. Thiram was less potent than Ziram at concentrations of 0.3–1 μM. Further, Zineb (1–3 μM) exhibited a very weak effect on FluoZin-3 fluorescence. These results are summarized in Fig. 1B.

3.2. Augmentation of Fluo-3 fluorescence by dithiocarbamate fungicides

Dithiocarbamate fungicides, such as Thiram and Ziram, were reported to increase intracellular Ca^{2+} levels in neuronal cells [21]. Therefore, their effects on Fluo-3 fluorescence, an indicator of intracellular Ca^{2+}, were tested. Incubation of cells with Ziram and Thiram (0.3–1 μM) slightly, but significantly, increased the