

Effects of Zn²⁺ chelators, DTPA and TPEN, and ZnCl₂ on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes

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ABSTRACT

Recently, we have revealed that trace Zn²⁺ partly attenuates Ca²⁺-dependent cell death induced by A23187, a calcium ionophore, in rat thymocytes. In this study, to see if Zn²⁺ attenuates the H₂O₂-induced cell death that is also Ca²⁺-dependent, the effects of ZnCl₂ and chelators for Zn²⁺ have been examined by using a flow-cytometer with propidium iodide. The incubation with H₂O₂ increased the cell lethality. Simultaneous application of ZnCl₂ greatly augmented the H₂O₂-induced increase in lethality. DTPA, a chelator for extracellular Zn²⁺, did not affect the increase in cell lethality by H₂O₂. However, the H₂O₂-induced increase in cell lethality was greatly attenuated by TPEN, a chelator for extracellular and intracellular Zn²⁺. Taken together, it may be likely that intracellular Zn²⁺ modifies the H₂O₂-induced cytotoxicity. However, it cannot be ruled out the possibility that TPEN chelates intracellular Fe²⁺, resulting in inhibiting the formation of hydroxyl radical from H₂O₂ that leads to an attenuation of H₂O₂ cytotoxicity.

Keywords: TPEN; DTPA; zinc; hydrogen peroxide; cytotoxicity

1. INTRODUCTION

Cell death induced by hydrogen peroxide (H₂O₂) in rat thymocytes is dependent on Ca²⁺ (Sakanashi *et al.*, 2008). Thus, the application of H₂O₂ increases intracellular Ca²⁺ concentration and the removal of external Ca²⁺ significantly attenuates the H₂O₂-induced cell death (Okazaki *et al.*, 1996; Nishizaki *et al.*, 2003; Sakanashi *et al.*, 2008). Sustained increase in intracellular Ca²⁺ concentration triggers either apoptotic or necrotic cell death (McConkey *et al.*, 1989; Azmi *et al.*, 1996; Berridge *et al.*, 1998; Orrenius *et al.*, 2003).

Recently, we have revealed that trace Zn²⁺ partly attenuates Ca²⁺-dependent cell death induced by A23187, a calcium ionophore, in rat thymocytes (Sakanashi *et al.*, 2009). Zinc itself modifies or induces cell death in a concentration-dependent manner (MacCabe *et al.*, 1993; Jiang *et al.*, 1995; Iguchi *et al.*, 1998; Kolenko *et al.*, 2001; Truong-

Tran *et al.*, 2001). Furthermore, zinc supplementation decreases oxidative stress induced by several types of compounds (DiSilvestro, 2000; Bao *et al.*, 2008; Szuster-Ciesielska *et al.*, 2009; Varghese *et al.*, 2009). Therefore, in this study, to see if Zn²⁺ attenuates the H₂O₂-induced cell death, the effects of ZnCl₂ and chelators for Zn²⁺, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and diethylenetriamine-N,N,N',N'',N''-pentaacetic acid (DTPA), on the cell death of rat thymocytes induced by H₂O₂ have been examined by using a flow-cytometer with propidium iodide.

Rat thymocytes were used for present study because of following reasons. First, the cell membranes of thymocytes remain intact because single cells can be prepared without enzymatic treatment. Second, the process of cell death is extensively studied in murine thymocytes (McConkey *et al.*, 1994; Tiso *et al.*, 1995; Rinner *et al.*, 1996; Winoto, 1997; Rennecke *et al.*, 2000; Quagliano and

Ronchetti, 2001; Ortiz *et al.*, 2001; Thompson *et al.*, 2003).

2. MATERIALS AND METHODS

2.1. Chemicals

H₂O₂ was purchased from Sumitomo Chemical Co. (Osaka, Japan). So-called chelators for Zn²⁺, TPEN and DTPA, were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). Propidium iodide was supplied from Molecular Probes Inc. (Eugene, Oregon, USA). Other chemicals (NaCl, CaCl₂, MgCl₂, KCl, glucose, HEPES, NaOH, and ZnCl₂) were also purchased from Wako Pure Chemicals.

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments in the University of Tokushima (No. 05279 for Y. Oyama).

The procedure to prepare cell suspension was similar to that previously reported (Chikahisa and Oyama, 1992; Chikahisa *et al.*, 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400-500 μ m with razor under an ice-cold condition (1-4°C). The slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3-7.4) to dissociate thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (a diameter of 10 μ m) to prepare the cell suspension (about 5 \times 10⁵ cells/ml). The beaker containing the cell suspension was water-bathed at 36°C for 1 hr before the experiment.

2.3. Experimental protocol

H₂O₂ was added to cell suspension (2 ml cell suspension in 10 ml test tube). To examine the effects of ZnCl₂, DTPA, and TPEN, the agents were respectively added to the suspension just before applying H₂O₂. The cell density was about 5 \times 10⁵ cells/mL. The cells were incubated with respective agent and hydrogen peroxide at 36°C for 2 hr under room air condition. The data acquisition of fluorescence from 2 \times 10³ cells by a flow cytometer required 10 sec at least.

2.4. Fluorescence measurements of cellular and membrane parameters

The method for measurement of cellular and membrane parameters, including forward scatter and side scatter, using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan)

and fluorescent probe was similar to those previously described (Chikahisa and Oyama, 1992; Chikahisa *et al.*, 1996). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition.

To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μ M. Since propidium stains dead cells, the measurement of propidium fluorescence from cells provides a clue to estimate the lethality. The fluorescence was measured at 2 min after the application of propidium iodide by a flow cytometer. Excitation wavelength for propidium was 488 nm and emission was detected at 600 \pm 20 nm.

2.5. Statistics

Values were expressed as the mean \pm standard deviation of 4 experiments. Statistical analysis was performed by using Tukey multivariate analysis. A *P* value of < 0.05 was considered significant.

3. RESULTS

3.1. Change of H₂O₂-induced increase in cell lethality of rat thymocytes by ZnCl₂, DTPA, or TPEN

There was no change in cell lethality of rat thymocytes after 2-3 h incubation with 10 μ M ZnCl₂, 10 μ M DTPA, 10 μ M TPEN, or 0.1 % DMSO as a solvent for TPEN. The application of 10 mM H₂O₂ time-dependently increased the population of cells exerting propidium fluorescence, presumably dead cells, indicating an increase in cell lethality. The cell lethality at 2 h after the start of H₂O₂ application ranged from 24.6 % to 44.0 %. Since prolonged incubation of cells with 10 mM H₂O₂ further increased the cell lethality, the values of cell lethality did not reach a steady state. DMSO at 0.1 % did not affect the H₂O₂-induced increase in cell lethality in the case of 2 h incubation.

As shown in Fig. 1, the incubation with 10 mM H₂O₂ for 2 h increased the population of cells exerting propidium fluorescence. Simultaneous application of 10 μ M ZnCl₂ greatly augmented the H₂O₂-induced increase in the population (Fig. 1). DTPA at 10 μ M, probably chelating extracellular Zn²⁺, did not affect the increase in cell lethality by 10 mM H₂O₂. However, the H₂O₂-induced increase in the population was greatly attenuated by 10 μ M TPEN, probably chelating extracellular and intracellular Zn²⁺ (Fig. 1). It is noted that normal Tyrode's solution with rat thymocytes contains 200-300 nM Zn²⁺ derived from the cells (Sakanashi *et al.*, 2009). Results are summarized in Fig. 2. ZnCl₂ significantly augmented the cytotoxicity of H₂O₂

while TPEN, but not DTPA, significantly attenuated it (Fig. 2). Thus, one may suggest that intracellular

Zn²⁺ has an essential role in H₂O₂-induced increase in lethality.

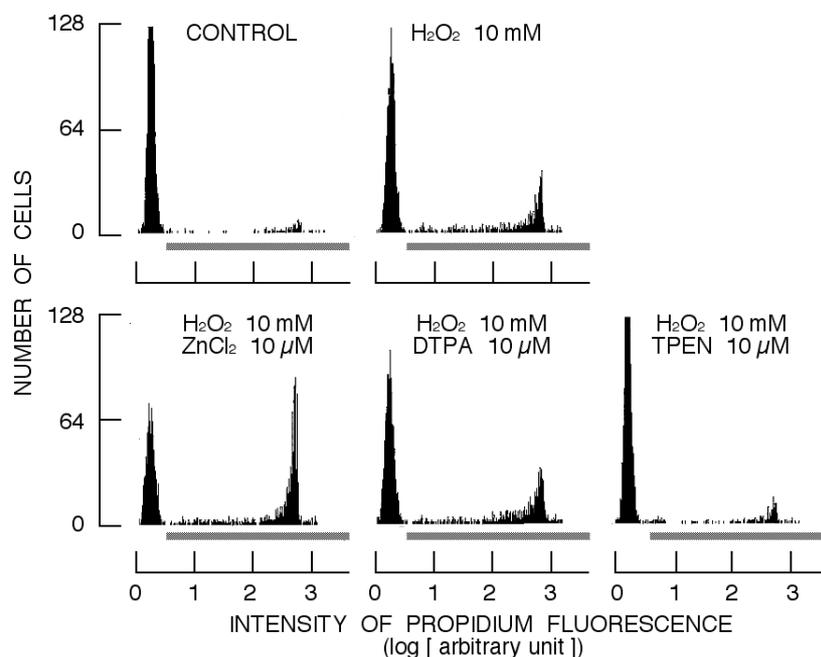


Fig.1. Effects of ZnCl₂, DTPA, and TPEN on H₂O₂-induced increase in population of cells exerting propidium fluorescence. Each histogram was constructed from 2 × 10³ cells. Bar under the histogram indicates the region of cells exerting propidium fluorescence, dead cells.

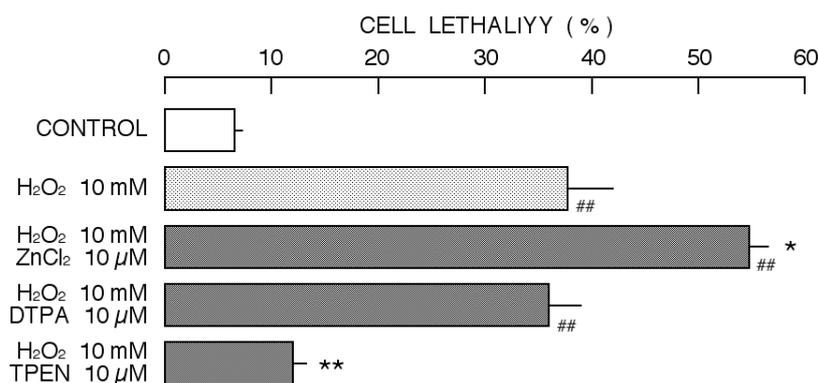


Fig. 2. Cell lethality of H₂O₂-treated cells in simultaneous presence of ZnCl₂, DTPA, or TPEN. Column and bar respectively indicate mean and standard deviation of four experiments. Symbol (##) near column indicates significant change (P < 0.01) to control group (CONTROL). Asterisks (* and **) show significant difference (P < 0.05 and P < 0.01, respectively) to the group of cells treated with H₂O₂ alone. It is noted that the difference between control group and the group of cells incubated with H₂O₂ and TPEN.

3.2. Change of H₂O₂-induced increase in population of shrunken cells by ZnCl₂, DTPA, or TPEN

Cytogram (forward scatter versus side scatter) was also affected by the incubation of cells with 10 mM H₂O₂ for 2 h. As shown in Fig. 3, the population of area S increased in the presence of H₂O₂. Simultaneous application of 10 mM H₂O₂ and 10 μM ZnCl₂ further increased the population of area S while 10 μM TPEN significantly attenuated the H₂O₂-

induced increase in the population (Fig. 3). DTPA at 10 μM did not significantly affect the population changed by 10 mM H₂O₂. Results are summarized in Fig. 4. ZnCl₂ significantly augmented the H₂O₂-induced change in cytogram while TPEN significantly attenuated it. DTPA did not affect the H₂O₂-induced change. Therefore, it is likely that the cell shrinkage is well-associated with the increase in cell lethality.

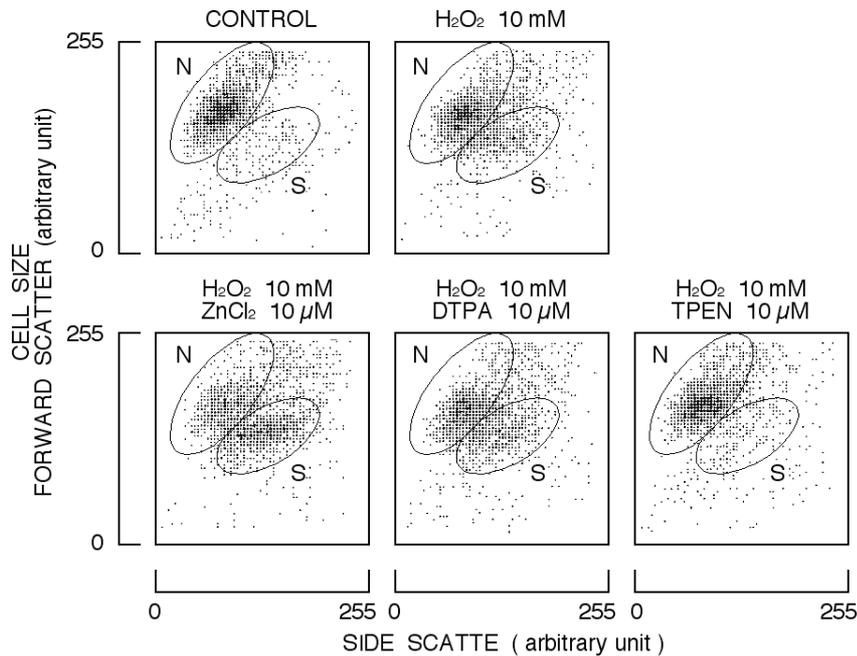


Fig. 3. Effect of H₂O₂ on cytogram (forward scatter versus side scatter) of rat thymocytes in simultaneous application of ZnCl₂, DTPA, or TPEN. Each cytogram was constructed with 2 × 10³ cells. Areas N and S indicate normal size cells and shrunken cells, respectively.

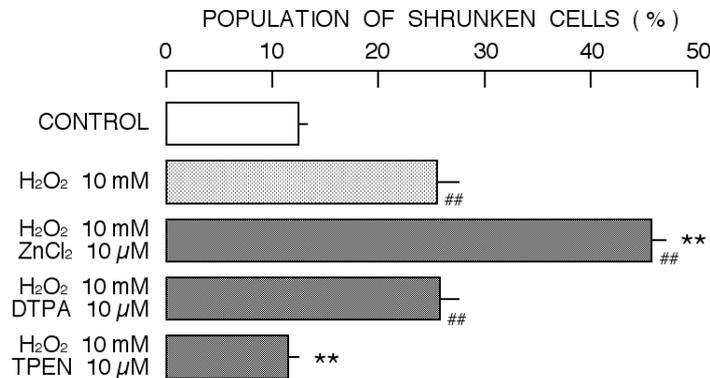


Fig. 4. Proportion of shrunken cells in the cells treated with H₂O₂ in simultaneous presence of ZnCl₂, DTPA, or TPEN. Column and bar respectively indicate mean and standard deviation of four experiments. Symbol (##) near column indicates significant change (P < 0.01) to the control group (CONTROL). Asterisk (**) shows significant difference (P < 0.01) to the group of cells treated with H₂O₂ alone.

4. DISCUSSION

DTPA is a chelator for extracellular Zn²⁺ while TPEN is one for both extracellular and intracellular Zn²⁺. Therefore, it is supposed that chelating intracellular Zn²⁺ is essential in attenuation of H₂O₂-induced cytotoxicity. The application of ZnCl₂ increases intracellular Zn²⁺ concentration (Matsui *et al.*, 2008). Thus, ZnCl₂ may augment H₂O₂-induced cytotoxicity by increasing intracellular Zn²⁺ concentration. Therefore, it may be presumably suggested that intracellular Zn²⁺ augments H₂O₂-induced cytotoxicity. Although this suggestion well corresponds to the results in this study, it is challenged by previous findings. First, the cell death induced by H₂O₂ in rat thymocytes is significantly dependent on Ca²⁺ (Okazaki *et al.*, 1996; Nishizaki *et al.*, 2003; Sakanashi *et al.*, 2008). Second, TPEN potentiates the Ca²⁺-dependent cytotoxicity of A23187 (Sakanashi *et al.*, 2009). Thus, it is also supposed that the application of TPEN augments the cytotoxicity of H₂O₂ because the H₂O₂ cytotoxicity is Ca²⁺-dependent. However, the cytotoxicity of H₂O₂ is dependent on Fe²⁺, rather than Ca²⁺ (Walker and Shah, 1991; Hiraishi *et al.*, 1993; Byler *et al.*, 1994; Lomonosova *et al.*, 1998). The combination of H₂O₂ and Fe²⁺ generates hydroxyl radical by Fenton reaction. Hydroxyl radical increases intracellular Ca²⁺ concentration (Dreher and Junod, 1995; Burlando and Viarengo, 2005). TPEN can chelate not only Zn²⁺ but also Fe²⁺. Therefore, it is supposed that TPEN can suppress the generation of hydroxyl radicals by chelating intracellular Fe²⁺,

resulting in attenuation of H₂O₂-induced cytotoxicity. Thus, the role of intracellular Zn²⁺ in H₂O₂ cytotoxicity cannot be strongly addressed at present although TPEN is known to be a chelator for intracellular Zn²⁺.

The H₂O₂-induced increase in cell lethality was augmented by adding ZnCl₂ (Figs. 1 and 2). In present study, we did not monitor the ZnCl₂-induced change in intracellular Zn²⁺ concentration during H₂O₂ exposure. However, the addition of ZnCl₂ may further elevate intracellular Zn²⁺ concentration since the application of H₂O₂ increases intracellular Zn²⁺ concentration (Hashimoto *et al.*, 2009). Excess increase in intracellular Zn²⁺ concentration leads to cell death (Kim *et al.*, 1999; Hamatake *et al.*, 2000; Itaka *et al.*, 2001). In this aspect, further study will be necessary to elucidate the role of intracellular Zn²⁺ in the augmentation of H₂O₂ cytotoxicity.

As to the population of shrunken cells (Figs. 3 and 4), one of parameters during an early stage of apoptosis, TPEN almost completely suppressed the H₂O₂-induced increase in shrunken cell population while ZnCl₂ greatly increased the population. Cell shrinkage is associated with an activation of Ca²⁺-dependent K⁺ channels in rat thymocytes (Horimoto *et al.*, 2006). Therefore, in the case of simultaneous presence of TPEN, it is unlikely that H₂O₂ increases intracellular Ca²⁺ concentration, as suggested above. On the other hand, the combination of ZnCl₂ and H₂O₂ may further increase not only intracellular Zn²⁺ but also intracellular Ca²⁺ concentration. In this aspect, further studies on intracellular Ca²⁺ and Zn²⁺ concentrations will be required.

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