Effects of Zn\textsuperscript{2+} chelators, DTPA and TPEN, and ZnCl\textsubscript{2} on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes

Hiroko Matsui\textsuperscript{a}, Yoko Sakashashi\textsuperscript{a}, Yumiko Nishimura\textsuperscript{b}, Takuya Kawanai\textsuperscript{c}, Yasuo Oyama\textsuperscript{c*}, Shiro Ishida\textsuperscript{a}, Yoshiro Okano\textsuperscript{d}

\textsuperscript{a} Faculty of Dental Science, Kyushu University, Fukuoka 812-8582, Japan
\textsuperscript{b} Okayama University Dental School, Okayama 700-8525, Japan
\textsuperscript{c} Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan
\textsuperscript{d} Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8512, Japan

* Corresponding author: Yasuo Oyama, Ph.D. E-mail: oyama@ias.tokushima-u.ac.jp

ABSTRACT

Recently, we have revealed that trace Zn\textsuperscript{2+} partly attenuates Ca\textsuperscript{2+}-dependent cell death induced by A23187, a calcium ionophore, in rat thymocytes. In this study, to see if Zn\textsuperscript{2+} attenuates the H\textsubscript{2}O\textsubscript{2}-induced cell death that is also Ca\textsuperscript{2+}-dependent, the effects of ZnCl\textsubscript{2} and chelators for Zn\textsuperscript{2+} have been examined by using a flow-cytometer with propidium iodide. The incubation with H\textsubscript{2}O\textsubscript{2} increased the cell lethality. Simultaneous application of ZnCl\textsubscript{2} greatly augmented the H\textsubscript{2}O\textsubscript{2}-induced increase in lethality. DTPA, a chelator for extracellular Zn\textsuperscript{2+}, did not affect the increase in cell lethality by H\textsubscript{2}O\textsubscript{2}. However, the H\textsubscript{2}O\textsubscript{2}-induced increase in cell lethality was greatly attenuated by TPEN, a chelator for extracellular and intracellular Zn\textsuperscript{2+}. Taken together, it may be likely that intracellular Zn\textsuperscript{2+} modifies the H\textsubscript{2}O\textsubscript{2}-induced cytotoxicity. However, it cannot be ruled out the possibility that TPEN chelates intracellular Fe\textsuperscript{3+}, resulting in inhibiting the formation of hydroxyl radical from H\textsubscript{2}O\textsubscript{2} that leads to an attenuation of H\textsubscript{2}O\textsubscript{2} cytotoxicity.

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

1. INTRODUCTION

Cell death induced by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in rat thymocytes is dependent on Ca\textsuperscript{2+} (Sakanashi et al., 2008). Thus, the application of H\textsubscript{2}O\textsubscript{2} increases intracellular Ca\textsuperscript{2+} concentration and the removal of external Ca\textsuperscript{2+} significantly attenuates the H\textsubscript{2}O\textsubscript{2}-induced cell death (Okazaki et al., 1996; Nishizaki et al., 2003; Sakanashi et al., 2008). Sustained increase in intracellular Ca\textsuperscript{2+} 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 Zn\textsuperscript{2+} partly attenuates Ca\textsuperscript{2+}-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 (McCabe 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 Zn\textsuperscript{2+} attenuates the H\textsubscript{2}O\textsubscript{2}-induced cell death, the effects of ZnCl\textsubscript{2} and chelators for Zn\textsuperscript{2+}, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and diethylenetriamine-N,N,N',N''-pentaacetic acid (DTPA), on the cell death of rat thymocytes induced by H\textsubscript{2}O\textsubscript{2} 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; Quaglino 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 by 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 μm 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 μm) to prepare the cell suspension (about 5 x 10⁶ 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 x 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 x 10⁵ 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 μM. 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 ± 20 nm.

2.5. Statistics

Values were expressed as the mean ± 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 μM ZnCl₂, 10 μM DTPA, 10 μM 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 μM ZnCl₂ greatly augmented the H₂O₂-induced increase in the population (Fig. 1). DTPA at 10 μM, 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 μM 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.
Effects of zinc and zinc chelators on H$_2$O$_2$ cytotoxicity

while TPEN, but not DTPA, significantly attenuated it (Fig. 2). Thus, one may suggest that intracellular Zn$^{2+}$ has an essential role in H$_2$O$_2$-induced increase in lethality.

**Fig. 1.** Effects of ZnCl$_2$, DTPA, and TPEN on H$_2$O$_2$-induced increase in population of cells exerting propidium fluorescence. Each histogram was constructed from 2 × 10$^4$ cells. Bar under the histogram indicates the region of cells exerting propidium fluorescence, dead cells.

**Fig. 2.** Cell lethality of H$_2$O$_2$-treated cells in simultaneous presence of ZnCl$_2$, 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 H$_2$O$_2$ alone. It is noted that the difference between control group and the group of cells incubated with H$_2$O$_2$ and TPEN.
3.2. Change of H$_2$O$_2$-induced increase in population of shrunken cells by ZnCl$_2$, DTPA, or TPEN

Cytogram (forward scatter versus side scatter) was also affected by the incubation of cells with 10 mM H$_2$O$_2$ for 2 h. As shown in Fig. 3, the population of area S increased in the presence of H$_2$O$_2$. Simultaneous application of 10 mM H$_2$O$_2$ and 10 μM ZnCl$_2$ further increased the population of area S while 10 μM TPEN significantly attenuated the H$_2$O$_2$-induced increase in the population (Fig. 3). DTPA at 10 μM did not significantly affect the population changed by 10 mM H$_2$O$_2$. Results are summarized in Fig. 4. ZnCl$_2$ significantly augmented the H$_2$O$_2$-induced change in cytogram while TPEN significantly attenuated it. DTPA did not affect the H$_2$O$_2$-induced change. Therefore, it is likely that the cell shrinkage is well-associated with the increase in cell lethality.

![Fig. 3. Effect of H$_2$O$_2$ on cytogram (forward scatter versus side scatter) of rat thymocytes in simultaneous application of ZnCl$_2$, DTPA, or TPEN. Each cytogram was constructed with 2 × 10$^5$ cells. Areas N and S indicate normal size cells and shrunken cells, respectively.](image)

![Fig. 4. Proportion of shrunken cells in the cells treated with H$_2$O$_2$ in simultaneous presence of ZnCl$_2$, 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 H$_2$O$_2$ alone.](image)
4. DISCUSSION

DTPA is a chelator for extracellular Zn\(^{2+}\) while TPEN is one for both extracellular and intracellular Zn\(^{2+}\). Therefore, it is supposed that chelating intracellular Zn\(^{2+}\) is essential in attenuation of H\(_2\)O\(_2\)-induced cytotoxicity. The application of ZnCl\(_2\) increases intracellular Zn\(^{2+}\) concentration (Matsui et al., 2008). Thus, ZnCl\(_2\) may augment H\(_2\)O\(_2\)-induced cytotoxicity by increasing intracellular Zn\(^{2+}\) concentration. Therefore, it may be presumably suggested that intracellular Zn\(^{2+}\) augments H\(_2\)O\(_2\)-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 H\(_2\)O\(_2\) in rat thymocytes is significantly dependent on Ca\(^{2+}\) (Okazaki et al., 1996; Nishizaki et al., 2003; Sakashiki et al., 2008). Second, TPEN potentiates the Ca\(^{2+}\)-dependent cytotoxicity of A23187 (Sakahashi et al., 2009). Thus, it is also supposed that the application of TPEN augments the cytotoxicity of H\(_2\)O\(_2\) because the H\(_2\)O\(_2\) cytotoxicity is Ca\(^{2+}\)-dependent. However, the cytotoxicity of H\(_2\)O\(_2\) is dependent on Fe\(^{2+}\), rather than Ca\(^{2+}\) (Walker and Shah, 1991; Hiraishi et al., 1993; Byler et al., 1994; Lomonosova et al., 1998). The combination of H\(_2\)O\(_2\) and Fe\(^{2+}\) generates hydroxyl radical by Fenton reaction. Hydroxyl radical increases intracellular Ca\(^{2+}\) concentration (Dreher and Junod, 1995; Burlando and Viarengo, 2005). TPEN can chelate not only Zn\(^{2+}\) but also Fe\(^{2+}\). Therefore, it is supposed that TPEN can suppress the generation of hydroxyl radicals by chelating intracellular Fe\(^{2+}\), resulting in attenuation of H\(_2\)O\(_2\)-induced cytotoxicity. Thus, the role of intracellular Zn\(^{2+}\) in H\(_2\)O\(_2\) cytotoxicity cannot be strongly addressed at present although TPEN is known to be a chelator for intracellular Zn\(^{2+}\).

The H\(_2\)O\(_2\)-induced increase in cell lethality was augmented by adding ZnCl\(_2\) (Figs. 1 and 2). In present study, we did not monitor the ZnCl\(_2\)-induced change in intracellular Zn\(^{2+}\) concentration during H\(_2\)O\(_2\) exposure. However, the addition of ZnCl\(_2\) may further elevate intracellular Zn\(^{2+}\) concentration since the application of H\(_2\)O\(_2\) increases intracellular Zn\(^{2+}\) concentration (Hashimoto et al., 2009). Excess increase in intracellular Zn\(^{2+}\) 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 Zn\(^{2+}\) in the augmentation of H\(_2\)O\(_2\) 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 H\(_2\)O\(_2\)-induced increase in shrunken cell population while ZnCl\(_2\) greatly increased the population. Cell shrinkage is associated with an activation of Ca\(^{2+}\)-dependen K\(^+\) channels in rat thymocytes (Horimoto et al., 2006). Therefore, in the case of simultaneous presence of TPEN, it is unlikely that H\(_2\)O\(_2\) increases intracellular Ca\(^{2+}\) concentration, as suggested above. On the other hand, the combination of ZnCl\(_2\) and H\(_2\)O\(_2\) may further increase not only intracellular Zn\(^{2+}\) but also intracellular Ca\(^{2+}\) concentration. In this aspect, further studies on intracellular Ca\(^{2+}\) and Zn\(^{2+}\) concentrations will be required.

REFERENCES


Hashimoto, E., Oyama, T.B., Oyama, K., Nishimura, Y., Oyama, T.M., Ueha-Ishibashi, T., Okano, Y., Oyama, Y., 2009. Increase in intracellular Zn\(^{2+}\) concentration by thimerosal in rat thymocytes: Intracellular Zn\(^{2+}\) release induced by oxidative...