Flow Cytometric Analysis of the H₂O₂-Induced Increase in Intracellular Ca²⁺ Concentration of Rat Thymocytes

Eisuke Okazaki¹, Lumi Chikahisa^{1,2,3}, Kaori Kanemaru¹ and Yasuo Oyama^{1,*}

¹Laboratory of Cell Signaling (Pharmacology), Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770, Japan

²Anticancer and Antimicrobials Research Laboratory, Taiho Pharmaceutical Co., Ltd., Tokushima 771-01, Japan

³Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., Hanno 357, Japan

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ABSTRACT—The effect of hydrogen peroxide (H₂O₂) on the intracellular Ca²⁺ concentration ([Ca²⁺]_i) of rat thymocytes was examined by a flow cytometer and two fluorescent dyes, fluo-3-AM and ethidium bromide, a dye impermeant to intact membranes, to characterize the H₂O₂-induced increase in [Ca²⁺]_i. H₂O₂ at concentrations greater than 30 μM dose-dependently increased the [Ca²⁺]_i of thymocytes which were not stained with ethidium. Removal of external Ca²⁺ greatly reduced the degree of H₂O₂-induced increase in [Ca²⁺]_i. However, H₂O₂ still increased the [Ca²⁺]_i under the external Ca²⁺-free condition. Diethylmaleate, which is known to produce a chemical depletion of cellular nonprotein thiol, significantly increased the [Ca²⁺]_i. Dithiothreitol, which is used to protect cellular nonprotein thiol, slightly decreased the [Ca²⁺]_i, but greatly reduced the H₂O₂-induced increase in [Ca²⁺]_i. Therefore, it is considered that H₂O₂ may increase the [Ca²⁺]_i through a mechanism related to the effects of H₂O₂ on the cellular nonprotein thiol.

Keywords: Flow cytometer, Nonprotein thiol, Hydrogen peroxide, Intracellular Ca²⁺, Oxidative stress

It is likely that the oxidative stress to mammalian cells induces an increase in the intracellular concentration of Ca²⁺ ([Ca²⁺]_i), a process that is proposed to be largely responsible for subsequent cell death or injury (1–4). Of all the biological oxidants, the effects of hydrogen peroxide (H₂O₂), a metabolite of superoxide anion, on the cellular functions and [Ca²⁺]_i have been intensively studied in several types of cells during the last decade (5–12). Therefore, the increase in the [Ca²⁺]_i may be a common feature of the cytotoxicity induced by H₂O₂. However, when the cells are dying or damaged during the exposure to H₂O₂, one can not rule out the possibility that the cells may lose a fluorescent Ca²⁺ indicator such as fluo-3 (13) because of compromised membranes, resulting in a decrease in fluorescence intensity that leads to an underestimation of [Ca²⁺]_i. Therefore, the fluorescent estimation of [Ca²⁺]_i should only be made in cells that can retain a fluorescent Ca²⁺ indicator. Flow cytometry offers clues for selecting the cells having intact membranes from the total cells and to measure the fluorescence of a Ca²⁺ indicator from the selected cells. In this study, in order to characterize the increase in the [Ca²⁺]_i induced by the ox-

idative stress and reveal its relation to the cellular redox status, we have examined the effects of H₂O₂ as a biological oxidant on fluo-3, 2',7'-dichlorofluorescein and 3-chloromethylfluorescein fluorescences only in individual cells that sufficiently retain the respective dyes by neglecting the cells that are dead (or damaged) or have compromised membranes using a flow cytometer and a combination of the respective dyes with ethidium bromide (14).

MATERIALS AND METHODS

Preparation

Experiments were performed on thymocytes dissociated from the thymus glands of 4-week-old Wistar rats (Nissin, Tokushima). Thymocytes were chosen for the experiments for the following reasons: First, the cells can be dissociated without treatment with proteolytic enzymes, such as collagenase and pronase, which may compromise the membranes of some cells during the digestion. The cell viability of dissociated thymocytes was greater than 97% under the control condition. Second, lymphocytes such thymocytes are very suitable for flow cytometric analysis because of their spherical shape, size and homogeneity. The technique for dissociation of rat

* To whom correspondence should be addressed.

thymocytes was a modification of that previously described by Chikahisa and Oyama (15). In brief, thymus glands were sliced at a thickness of 400 to 500 μm . Thereafter, the slices were gently triturated in chilled Tyrode's solution to dissociate single thymocytes. Tyrode's solution containing dissociated thymocytes was passed through a mesh (diameter of 53 μm) to remove residues.

Fluorescence measurements and analyses

To estimate the $[\text{Ca}^{2+}]_i$ from only live thymocytes, two fluorescent probes, fluo-3-AM (Dojindo Laboratory, Kumamoto) and ethidium bromide (Katayama Chemical Industries, Osaka) were used. The respective dye was added into the cell suspension to achieve a final concentration of 500 nM for fluo-3-AM or 5 μM for ethidium bromide (14). Fluo-3-AM, an esterase substrate of fluo-3, passively crosses the membranes of live thymocytes and is then converted by cellular esterase to fluo-3, which is retained by live thymocytes with intact plasma membranes. Fluo-3 is predominantly used for monitoring the $[\text{Ca}^{2+}]_i$ because this dye would be fluorescent if bound to intracellular Ca^{2+} (16). Ethidium, which is highly impermeant to intact membrane, can not stain live cells (13). Therefore, live thymocytes show the fluo-3 fluorescence while there is no fluo-3 fluorescence in dead thymocytes. In contrast, dead thymocytes show ethidium fluorescence because ethidium can stain thymocytes that are dead or have compromised membranes. Therefore, the simultaneous measurement of fluo-3 and ethidium fluorescences from thymocytes would enable us to estimate the $[\text{Ca}^{2+}]_i$ from cells with intact membranes (14). Before any measurement of fluo-3 fluorescence, thymocytes were incubated with fluo-3-AM for 60 min. Fluorescence from thymocytes incubated with ethidium bromide was measured at 2–3 min after the dye application because ethidium slightly stained live cells during a prolonged exposure to this dye and 2–3 min was enough time to stain dead cells. Dual measurements for both fluo-3 and ethidium fluorescences from thymocytes were made by a flow cytometer (Cyto ACE-150; Japan Spectroscopic Co., Tokyo). The excitation wavelength for both fluo-3 and ethidium was 488 nm produced by an argon laser. Emissions were detected at wavelengths of 530 ± 20 nm for fluo-3 and 600 ± 20 nm for ethidium. Fluorescence cytograms (fluo-3 fluorescence intensity versus ethidium fluorescence intensity) obtained from a programmed number of thymocytes was analyzed by software (Jasco Ver.3XX, Japan Spectroscopic Co.) and a personal computer (PC-9801RX; NEC, Tokyo). The fluo-3 fluorescence was measured during 60–180 min after the dye application because the intensity of fluo-3 fluorescence was relatively constant during this period. Tentative calibra-

tion of fluo-3 fluorescence for the $[\text{Ca}^{2+}]_i$ was made by a method using ionomycin and Mn^{2+} (16). Thus, the $[\text{Ca}^{2+}]_i$ was estimated by the following equation (16): $[\text{Ca}^{2+}]_i = K_d(F - F_{\min}) / (F_{\max} - F)$, where K_d is 400 nM at vertebrate ionic strength, $F_{\max}$ represents the maximum fluorescence intensity from the Ca^{2+} -saturated cellular dye, $F_{\min}$ represents the minimum intensity predicted from the metal-free dye fluorescence and background fluorescence, and F is the fluorescence intensity obtained during the experiment. All fluorescence intensities were obtained from the mean intensity of the fluo-3 fluorescence histogram constructed from 5000 thymocytes (17). To estimate the cellular redox status of thymocytes, two fluorescent dyes, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probe, Inc., Eugene, OR, USA) and 3-chloromethylfluorescein diacetate (CMF-DA; Molecular Probe, Inc.), were used (18–20). DCFH-DA and CMF-DA, which both can diffuse into the thymocytes, are respectively hydrolyzed to DCFH and CMF by cellular esterase. DCFH is oxidized by cellular peroxides including H_2O_2 to DCF, a fluorescent form (18, 19). The concentration of DCFH-DA was 30 μM or 100 μM , and the DCF fluorescence was measured at least 60 min after application of DCFH-DA (19). CMF-DA is used to estimate the cellular level of nonprotein thiols (20). Thus, CMF fluorescence of thymocytes can be attenuated by agents that decrease the level of cellular nonprotein thiols (mainly glutathione), such as *N*-ethyl maleimide, diethylmaleate and buthione sulfoxamine, suggesting that CMF fluorescence can be utilized for estimating the change in cellular level of nonprotein thiols (21). The measurement of CMF fluorescence was performed at 30–60 min after the application of 1 μM CMF-DA, because during this time period, the CMF fluorescence attains its peak intensity. Thus, the measurement of DCF and CMF fluorescences provides information about the cellular redox status of thymocytes. The excitation wavelength for both dyes was 488 nm. Emission was detected at the wavelength of 530 ± 20 nm. Statistical analyses was performed by the two sample *t*-test; A *P* value of < 0.05 was considered to be significant.

Chemicals

Hydrogen peroxide was purchased from Sumitomo Chemicals (Osaka). Ionomycin (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in ethanol. Ethanol as a solvent at a final concentration of 0.3% or less did not affect any fluorescence measurement. All stock solutions were stored in the freezer and used within 2 weeks after preparation. Other chemical reagents except for fluorescent dyes were obtained from Katayama Chemical Industries (Osaka).

Fig. 1. Flow-cytometric measurements of fluo-3 and ethidium fluorescence from a programmed number of rat thymocytes (5000 cells) in the cell suspension to which 500 nM fluo-3-AM and 5 μM ethidium bromide were added. Panels show the fluorescence cytograms, fluo-3 fluorescence (abscissa) versus ethidium fluorescence (ordinate), before (left panel, Control) and 2 min after application of 50 μM digitonin (right panel, 2 min).

RESULTS

Effects of H_2O_2 on fluo-3 fluorescence obtained from thymocytes with intact membranes

There was a large population of cells stained predominantly with fluo-3, but not with ethidium, in the control cytogram (Fig. 1), while a large population of cells was predominantly stained with ethidium after lysis of the cells with 50 μM digitonin, which released fluo-3 and permitted ethidium to permeate the membranes. Digitonin was reported to release essentially all of the lactic dehydrogenase, a standard cytosolic marker (16).

Fig. 2. Effects of 1 mM H_2O_2 on the histograms of ethidium fluorescence obtained from a programmed number of rat thymocytes (4000 cells) in the cell suspension. Left panel: superimposed histograms obtained before (Control) and after application of 50 μM digitonin (Digitonin, 50 μM). Right panel: histogram (H_2O_2 1 mM) obtained at 90 min after the start of the treatment with 1 mM H_2O_2 . The histogram was superimposed with that obtained after application of 50 μM digitonin. Dotted lines under the histograms indicate the cells possessing the ethidium fluorescence.

Therefore, the cells stained predominantly with ethidium in the cytogram are dead or have compromised membranes (13). As shown in the ethidium fluorescence histograms of Fig. 2, the cell suspension contained the cells exhibiting ethidium fluorescence that were dead or had compromised membranes (13). We can not rule out the possibility that such cells may lose the fluorescent Ca^{2+} indicator because of compromised membranes. Therefore, in this study, the fluo-3 fluorescence histograms were obtained from only thymocytes that were not stained with ethidium. H_2O_2 at a concentration of 300 μM shifted the fluo-3 fluorescence histogram towards higher intensities in a time-dependent manner (Fig. 3). Calculated $[\text{Ca}^{2+}]_i$ from the mean intensity of the fluorescence histogram obtained from thymocytes increased time-dependently during a continued exposure to 300 μM H_2O_2 (Fig. 4). However, a prolonged exposure of thymocytes to H_2O_2 at the concentrations of 100 μM or higher produced a great decrease in the cell viability of thymocytes (21). Therefore, the dose-dependent effect of H_2O_2 on the $[\text{Ca}^{2+}]_i$ was examined at 90 min after application of H_2O_2 at concentrations ranging from 1 to 3 μM (Fig. 5). H_2O_2 started to increase the $[\text{Ca}^{2+}]_i$ at a concentration of 10 μM . Increasing the concentration of H_2O_2 produced a further increase in the $[\text{Ca}^{2+}]_i$ of thymocytes. Drastic increases in the $[\text{Ca}^{2+}]_i$ were seen at the concentrations of 1 and 3 mM. As shown in Fig. 5, the amount of $[\text{Ca}^{2+}]_i$ increased by 1 mM H_2O_2 greatly varied from cell to cell, resulting in a large S.D. of the mean $[\text{Ca}^{2+}]_i$, although their mean value was about 2 μM . Although the $[\text{Ca}^{2+}]_i$ of thymocytes exposed to 3 mM H_2O_2 could not be estimated from the fluo-3 fluorescence in many cells because of the saturated intensity of fluo-3 fluorescence, the $[\text{Ca}^{2+}]_i$ of thymocytes exposed to 3 mM H_2O_2 was thought to be greater than 3 μM . Removal of external Ca^{2+} by substitution of equimolar Mg^{2+} and addition of

Fig. 3. The time-dependent changes in the fluorescence histogram, the intensity of fluo-3 fluorescence (abscissa) versus the number of cells (ordinate), at 30 min (left panel), 60 min (middle panel) and 90 min (right panel) after application of 300 μ M H_2O_2 . Flow-cytometric measurement of fluo-3 fluorescence from a programmed number (5000 cells) of dissociated thymocytes that were not stained with ethidium. CONT. and H_2O_2 in the individual panels indicate the control histogram and the histogram in the continued presence of 300 μ M H_2O_2 , respectively.

2 mM EDTA greatly reduced the increase in the $[Ca^{2+}]_i$ induced by 1 mM H_2O_2 (Fig. 6), indicating the dependence of the H_2O_2 -induced increase in $[Ca^{2+}]_i$ on the external Ca^{2+} . However, H_2O_2 still increased the $[Ca^{2+}]_i$ under the external Ca^{2+} -free condition, suggesting that H_2O_2 may affect the intracellular Ca^{2+} stores.

Effects of diethylmaleate and dithiothreitol on the fluo-3 fluorescence of thymocytes

H_2O_2 at the concentrations of 300 μ M or more greatly attenuated the intensity of 5-chloromethylfluorescein

(CMF) fluorescence, which presumably represents the cellular level of nonprotein thiols (20), in rat thymocytes (21). Therefore, the level of cellular nonprotein thiols may be linked to the H_2O_2 -induced increase in the $[Ca^{2+}]_i$ of rat thymocytes. To test this possibility, the effects of diethylmaleate (DEM) and dithiothreitol (DTT) on the fluo-3 fluorescence were examined. DEM at a concentration of 5 mM was used to induce a chemical depletion of cellular nonprotein thiols (22). DEM increased the $[Ca^{2+}]_i$ to a level greater than 400 nM (Fig. 7). DTT at a concentration of 10 mM was also used as a sulfhydryl-

Fig. 4. Time-dependent change in the mean concentration of intracellular Ca^{2+} after the start of the application of 300 μ M H_2O_2 . The value at zero min in the abscissa indicates the control before application of H_2O_2 . Dotted lines indicate the control level. Each symbol and bar show the average and S.D. of six experiments, respectively.

Fig. 5. Dose-dependent effect of H_2O_2 on the mean concentration of intracellular Ca^{2+} of thymocytes. Effects were examined at 90 min after application of H_2O_2 at the respective concentrations. Each symbol and bar, respectively, indicate the average of the mean concentration and S.D. of six experiments. #Note that the $[\text{Ca}^{2+}]_i$ of thymocytes exposed to 3 mM H_2O_2 could not be estimated from the fluo-3 fluorescence because of the saturation of fluorescence intensity in many cells, and $[\text{Ca}^{2+}]_i$ was tentatively estimated to be greater than 3 μM .

protecting agent. DTT slightly decreased the control level of $[\text{Ca}^{2+}]_i$. DTT attenuated the H_2O_2 -induced increase in the $[\text{Ca}^{2+}]_i$. Thus, H_2O_2 at a concentration of 1 mM increased $[\text{Ca}^{2+}]_i$ to greater than 3 μM , while the pretreatment of thymocytes with DTT significantly reduced the efficacy of 1 mM H_2O_2 in increasing $[\text{Ca}^{2+}]_i$. Therefore, the intracellular level of nonprotein thiols in thymocytes seems to be related to the H_2O_2 -induced increase in

$[\text{Ca}^{2+}]_i$. To estimate the redox status of thymocytes, we examined the effects of 1 mM H_2O_2 and 5 mM DEM on the respective fluorescence obtained from thymocytes incubated with 30 μM DCFH-DA or 1 μM CMF-DA. As shown in Fig. 8, 5 mM DEM augmented the DCF fluorescence, indicating the increase in the oxidation of cellular DCFH by cellular peroxide, including H_2O_2 (18, 19), and attenuated the intensity of CMF fluorescence,

Fig. 6. Effect of 1 mM H_2O_2 on the mean intracellular Ca^{2+} concentration under the control (open column) and nominal external Ca^{2+} -free (filled column) conditions. Effects of H_2O_2 were examined at 90 min after application. Each column and bar, respectively, indicate the average and S.D. of four experiments. Asterisks indicate significant changes as compared with the control (* $P < 0.05$ and ** $P < 0.005$).

Fig. 7. Effects of 5 mM diethylmaleate (DEM) and 10 mM dithiothreitol (DTT) on the mean intracellular Ca^{2+} concentration of thymocytes. Effects were examined at 90 min after the respective applications. Each column and bar, respectively, indicate the average and S.D. of four experiments. Asterisks indicate significant changes between bars as indicated with arrows (* $P < 0.01$ and ** $P < 0.001$).

Fig. 8. Effects of H_2O_2 and diethylmaleate (DEM) on the fluorescences of 2',7'-dichlorofluorescein (DCF, upper panel) and 5-methylchlorofluorescein (CMF, lower panel) of thymocytes. Effects on CMF fluorescence were examined at 90–120 min after the respective applications (H_2O_2 or diethylmaleate) to ensure that the intensity of CMF fluorescence was at its peak. It was 90 min in the case of DCF fluorescence. Each column and bar, respectively, indicate the mean value of intensity relative to the respective control and S.D. of three experiments. The dead cells were removed from the measurements of DCF and CMF fluorescence by identifying them with ethidium bromide that stained dead cells. Asterisks indicate significant changes as compared with the control (* $P < 0.01$, ** $P < 0.005$).

suggesting the decrease in cellular level of nonprotein thiol (20). H_2O_2 exerted similar effects on DCF and CMF fluorescences. Its effects were much more profound than those of DEM, and the potency of H_2O_2 in increasing the $[\text{Ca}^{2+}]_i$ was much greater than that of DEM.

DISCUSSION

Implications of dual measurement of fluo-3 and ethidium fluorescences for estimating intracellular Ca^{2+} of thymocytes

The intensity of ethidium fluorescence was measured

concomitantly with fluo-3 fluorescence because ethidium is highly impermeant to the membranes of intact thymocytes. Ethidium is permeant following membrane damaging or membrane disruption, resulting in augmentation of cellular ethidium fluorescence. Fluo-3, which is converted from fluo-3-AM by a cellular esterase, is fluorescent when it binds to intracellular Ca²⁺ if thymocytes are intact. Thymocytes with compromised membranes are unable to retain fluo-3, resulting in decreased intensity of fluo-3 fluorescence that leads to underestimation of [Ca²⁺]_i. Dual measurement of fluo-3 and ethidium fluorescences can determine live and dead (damaged) cells as well as the [Ca²⁺]_i of live cells. It enables us to accurately monitor [Ca²⁺]_i only from thymocytes that are thought to retain fluo-3 by neglecting thymocytes stained with ethidium from the total thymocytes.

H₂O₂-induced increase in [Ca²⁺]_i

H₂O₂ dose- and time-dependently increased the [Ca²⁺]_i of thymocytes (Figs. 3–5). Furthermore, the increase in [Ca²⁺]_i induced by H₂O₂ was largely dependent on the presence of external Ca²⁺ because the H₂O₂-induced increase in the [Ca²⁺]_i was greatly attenuated under the external Ca²⁺-free condition (Fig. 6). Since the fluo-3 fluorescence was measured in the thymocytes that were not stained with ethidium, the membranes of thymocytes used for monitoring [Ca²⁺]_i were considered to retain their integrity. Therefore, it is likely that H₂O₂ elevates the [Ca²⁺]_i by increasing the membrane permeability of Ca²⁺, but not by compromising the membranes of thymocytes. DTT (10 mM), a thiol-protecting agent, reduced the amount of H₂O₂-induced increase in [Ca²⁺]_i, while 5 mM DEM, a chemical reagent for cellular non-protein thiols, increased the [Ca²⁺]_i (Fig. 7). Both H₂O₂ (1 mM) and DEM (5 mM) greatly attenuated the CMF fluorescence representing the cellular level of nonprotein thiols, mainly glutathione, and augmented the DCF fluorescence (Fig. 8). Therefore, it seems plausible that H₂O₂ increases the [Ca²⁺]_i through a mechanism related to the effect of H₂O₂ on the cellular redox state. H₂O₂ still increased the [Ca²⁺]_i of thymocytes in the nominal absence of external Ca²⁺ (Fig. 6). H₂O₂ has been shown to release Ca²⁺ from intracellular organelles, such as the endoplasmic reticulum (23) and mitochondria (24, 25), for the purpose of maintaining the homeostasis of intracellular Ca²⁺. It is likely that the H₂O₂-induced increase in the [Ca²⁺]_i is one of common features in the toxicity of the oxidative stress (5–12). In dissociated rat cerebellar neurons whose [Ca²⁺]_i is increased by the application of H₂O₂ (Y. Oyama et al., unpublished data), the calcium ionophore ionomycin greatly increased the intensity of DCF fluorescence in a Ca²⁺-dependent manner (26–28), suggesting the Ca²⁺-induced increase in H₂O₂ formation.

There is a possibility that the excessive influx of Ca²⁺ into the cells augments the formation of H₂O₂, which further increases the [Ca²⁺]_i.

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