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ORIGINAL PAPER

Putative role of intracellular Zn^{2+} release during oxidative stress: a trigger to restore cellular thiol content that is decreased by oxidative stress

Akio Kinazaki · Hongqin Chen · Kazuki Koizumi · Takuya Kawanai · Tomohiro M. Oyama · Masaya Satoh · Shiro Ishida · Yoshiro Okano · Yasuo Oyama

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Abstract Although the ability of zinc to retard the oxidative process has been recognized for many years, zinc itself has been reported to induce oxidative stress. In order to give some insights into elucidating the role of intracellular Zn^{2+} in cells suffering from oxidative stress, the effects of *N*-ethylmaleimide (NEM) and $ZnCl_2$ on cellular thiol content and intracellular Zn^{2+} concentration were studied by use of 5-chloromethylfluorescein diacetate (5-CMF-DA) and FluoZin-3 pentaacetoxymethyl ester (FluoZin-3-AM) in rat thymocytes. The treatment of cells with NEM attenuated 5-CMF fluorescence and augmented FluoZin-3 fluorescence in a dose-dependent manner. These NEM-induced phenomena were observed under external Zn^{2+}-free conditions. Results suggest that NEM decreases cellular thiol content and induces intracellular Zn^{2+} release. Micromolar $ZnCl_2$ dose-dependently augmented both FluoZin-3 and 5-CMF fluorescences, suggesting that the elevation of intracellular Zn^{2+} concentration increases cellular thiol content. Taken together, it is hypothesized that intracellular Zn^{2+} release during oxidative stress is a trigger to restore cellular thiol content that is decreased by oxidative stress.

A. Kinazaki · S. Ishida · Y. Okano
Graduate School of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan

H. Chen · K. Koizumi · T. Kawanai · M. Satoh · Y. Oyama (✉)
Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan
e-mail: oyama@ias.tokushima-u.ac.jp

T. M. Oyama
Hakata Clinic, Medical Co. LTA Clinical Pharmacology Center, Fukuoka, Japan

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Introduction

Although the ability of zinc to retard the oxidative process has been recognized for many years (see for reviews [1, 2]), the mechanism by which zinc reduces oxidative stress is not well understood. The results obtained under zinc-deficient conditions show that zinc deprivation generally results in an increase in susceptibility to oxidative stress [3–6]. Zinc significantly increases glutathione level in ARPE-19 cells through the induction of a de novo synthesis pathway [7] and protects from peroxide-induced cell death via increasing transcription of catalytic subunit of glutamate-cysteine ligase and glutathione concentration in primary rat endothelial cells [8]. Zinc supplementation to human subjects lowers plasma markers of oxidative stress [9–11]. However, it is proposed that zinc exposure results in mitochondrial injury and that reactive oxygen species are involved in zinc cytotoxicity [12–14]. Furthermore, under oxidative stress induced by hydrogen peroxide, zinc exerts cytotoxic action by excessive increase in intracellular Zn^{2+} concentration [15]. Chelation of intracellular Zn^{2+} protects the cells suffering from hydrogen peroxide at lethal concentrations [16]. Therefore, there may be complicated relationship between zinc and oxidative stress.

Intracellular Zn^{2+} makes a complex with the thiol group of protein and nonprotein [17–19]. Modification from thiol to disulfide by oxidative stress releases Zn^{2+} from protein and nonprotein [20, 21]. We show negative correlation between intracellular Zn^{2+} concentration (the intensity of FluoZin-3 fluorescence) and cellular content of nonprotein thiols (the intensity of 5-CMF fluorescence) in the cells treated with

thimerosal [22], methylmercury [23], and tri-*n*-butyltin [24]. These organometallic compounds seem to increase intracellular Zn^{2+} concentration by decreasing cellular content of nonprotein thiols. Since organometallic compounds provide a source of nucleophilic carbon atoms, their reaction is not specific for thiols and these compounds may not be suitable to emphasize the correlation between intracellular Zn^{2+} concentration and cellular thiol content. *N*-Ethylmaleimide (NEM) is an excellent reagent for thiol-selective modification, quantitation, and analysis and is widely used to prove a functional role of thiol group in enzymology [25–27]. NEM can be used to examine the correlation. In this study, we first examined the correlation between 5-CMF fluorescence intensity (cellular thiol content) and FluoZin-3 fluorescence intensity (intracellular Zn^{2+} concentration) by the use of NEM. As described above, the effect of zinc on cellular glutathione content is controversial. The application of micromolar $ZnCl_2$ greatly augments FluoZin-3 fluorescence, indicating a $ZnCl_2$-induced increase in intracellular Zn^{2+} concentration [28]. Therefore, second, the correlation between FluoZin-3 fluorescence intensity and 5-CMF fluorescence intensity was examined by the use of $ZnCl_2$. Such experiments may give some insights into elucidainge physiological and/or pathological roles of intracellular Zn^{2+} in the cells suffering from oxidative stress.

Materials and methods

Chemicals

Chelators for Zn^{2+}, *N*,*N*,*N*′,*N*′-tetrakis[2-pyridylmethyl] ethylenediamine (TPEN) and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), were obtained from the Dojin Chemical Laboratory (Kumamoto, Japan). FluoZin-3-AM, 5-CMF-DA, and propidium iodide were products of Molecular Probes (Eugene, OR, USA). Other chemicals (NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and $ZnCl_2$) were purchased from Woko Pure Chemicals (Osaka, Japan).

Animals and cell preparation

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

The procedure to prepare the cell suspension was similar to that previously reported [29, 30]. In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400–500 μm with a razor under a cold condition (3–4°C). The slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust the pH to 7.3–7.4) to dissociate thymocytes. Then, the Tyrode's solution containing the cells was passed through a mesh (diameter 10 μm) to prepare the cell suspension. The beaker containing the cell suspension was water-bathed at 36–37°C for 1 h before the experiment. Although the chemical composition of Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM zinc derived from the cell preparation [31].

Rat thymocytes were used for the present study for the 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 has been extensively studied in murine thymocytes [32–35].

Fluorescence measurements of cellular and membrane parameters

The methods for measurements of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described [28–30]. The fluorescence was analyzed by JASCO software (Ver.3XX; JASCO). There was no fluorescence from reagents used in this study, except for fluorescent probes, under our experimental conditions.

To assess cell lethality, propidium iodide was added to the 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.

FluoZin-3-AM [36] was used as an indicator for intracellular Zn^{2+}. The cells were incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements to estimate the change in intracellular Zn^{2+} concentration of rat thymocytes with intact membranes. FluoZin-3 fluorescence was measured from the cells that were not stained with 5 μM propidium iodide [28]. Excitation wavelength for FluoZin-3 was 488 nm and emission was detected at 530 ± 20 nm.

5-CMF-DA was used to monitor the change in cellular content of nonprotein thiols [30]. The cells were incubated with 1 μM 5-CMF-DA for 30 min before any fluorescence measurements. 5-CMF fluorescence was measured from the cells that were not stained with 5 μM propidium iodide. Excitation wavelength for 5-CMF was 488 nm and emission was detected at 530 ± 15 nm.

Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed with

Turkey multivariate analysis. A P value of <0.05 was considered significant.

Results

Change in 5-CMF fluorescence by NEM

N-ethylmaleimide has been used to decease (or deplete) the cellular content of nonprotein thiols in several types of preparations [37–40]. As shown in Fig. 1a, the incubation with 10 or 30 μM NEM for 90 min shifted the histogram of 5-CMF fluorescence in the direction of lower intensity, indicating NEM-induced decrease in cellular content of nonprotein thiols, presumably glutathione [30]. NEM at concentrations ranging from 3 to 100 μM significantly attenuated 5-CMF fluorescence in a concentration-dependent manner when the cells were incubated with NEM for 90 min (Fig. 1b), indicating that NEM concentration-dependently induces the decrease (or depletion) of cellular thiol content.

Change in FluoZin-3 fluorescence by NEM

To see if the change in cellular thiol content affects the intracellular Zn^{2+} concentration, the effect of NEM on FluoZin-3 fluorescence was examined. The incubation with 10 or 30 μM NEM for 90 min shifted the histogram of FluoZin-3 fluorescence in the direction of higher intensity (Fig. 2a), indicating NEM-induced increase in intracellular Zn^{2+} concentration. As shown in Fig. 2b, NEM at concentrations of 10–100 μM significantly augmented FluoZin-3 fluorescence in a concentration-dependent manner. Results suggest that NEM at 10–100 μM concentration-dependently increases intracellular Zn^{2+} concentration.

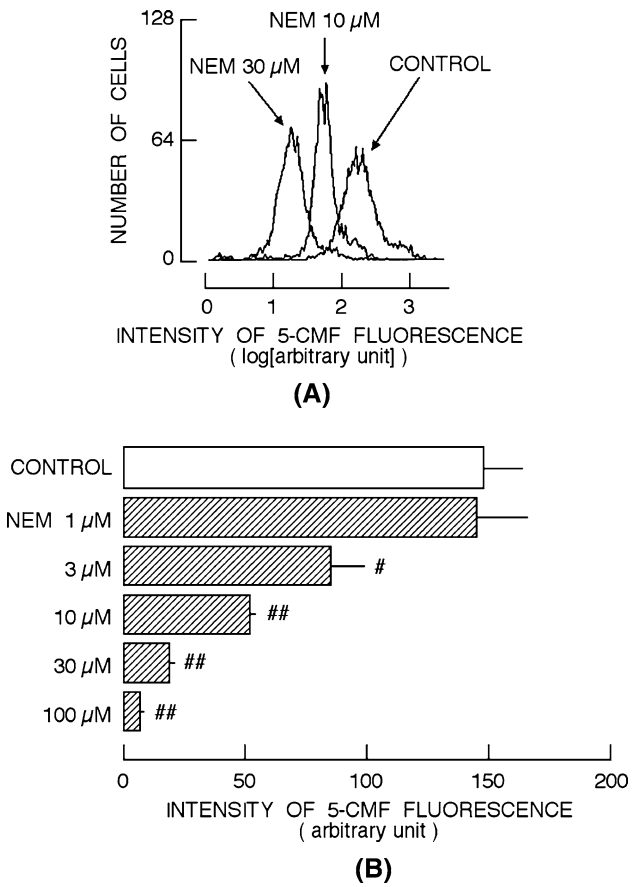


Fig. 1 Change in 5-CMF fluorescence intensity by NEM. **a** NEM-induced shift of 5-CMF fluorescence histogram. 5-CMF fluorescence was measured from cells that were not stained with propidium iodide. Each histogram was constructed with 2,500 cells. **b** Concentration-dependent attenuation of 5-CMF fluorescence by NEM. *Columns* and *bars* indicate means and standard deviations, respectively, of five experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significant differences compared with control

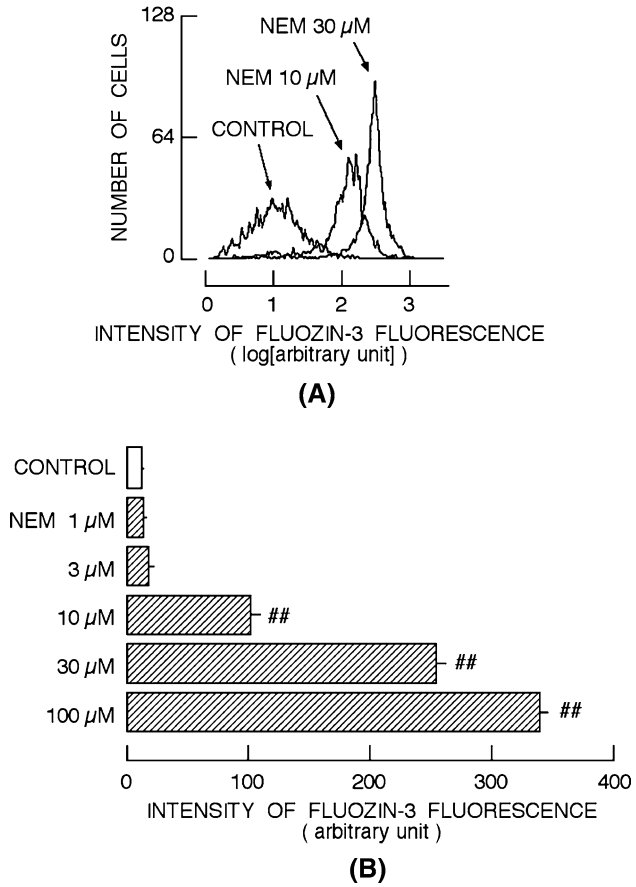


Fig. 2 Change in FluoZin-3 fluorescence intensity by NEM. **a** NEM-induced shift of FluoZin-3 fluorescence histogram. FluoZin-3 fluorescence was measured from cells that were not stained with propidium iodide. Each histogram was constructed with 2,500 cells. **b** Concentration-dependent augmentation of FluoZin-3 fluorescence by NEM. *Columns* and *bars* indicate means and standard deviations, respectively, of five experiments. $^{\#\#}P < 0.01$ significant difference compared with control

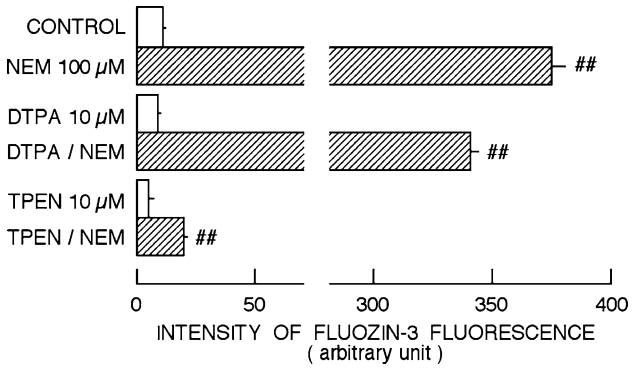


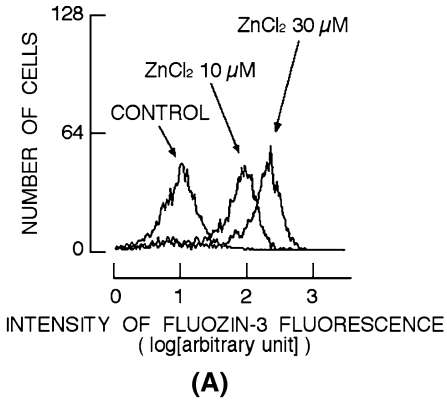
Fig. 3 NEM-induced augmentation of FluoZin-3 fluorescence in cells treated with Zn^{2+}-chelators, DPTA and TPEN. *Columns* and *bars* indicate means and standard deviations, respectively, of five experiments. $^{##}P < 0.01$ significant difference compared with control

Effects of Zn^{2+}-chelators on NEM-induced change in FluoZin-3 fluorescence

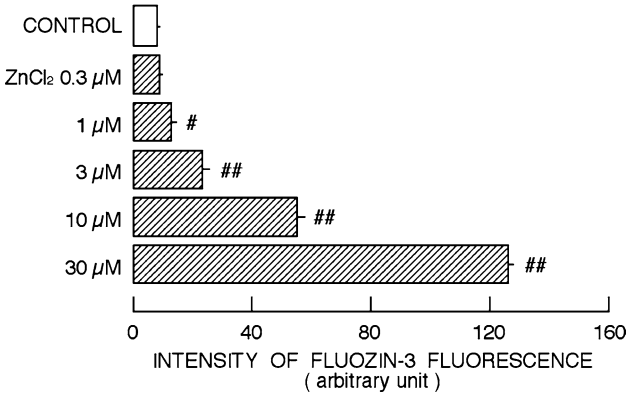
To reveal the source of Zn^{2+} for NEM-induced increase in intracellular Zn^{2+} concentration, NEM-induced augmentation of FluoZin-3 fluorescence was compared in the cells treated with Zn^{2+}-chelators, DTPA and TPEN (Fig. 3). In the cells incubated with 10 μM DTPA, a chelator for extracellular Zn^{2+}, NEM at 100 μM greatly augmented FluoZin-3 fluorescence from 9.3 ± 0.4 (arbitrary unit, mean ± SD of five experiments) to 341.4 ± 9.8. The augmentation by NEM under the condition without DTPA was from 11.3 ± 0.2 to 374.8 ± 5.7. Thus, the chelation of external Zn^{2+} by DTPA slightly attenuated NEM-induced augmentation of FluoZin-3 fluorescence. In the presence of 10 μM TPEN, a membrane-permeable Zn^{2+} chelator, NEM augmented FluoZin-3 fluorescence from 5.6 ± 0.1 to 20.0 ± 0.4. Thus, TPEN drastically decreased NEM-induced augmentation of FluoZin-3 fluorescence. Results indicate that the NEM-induced augmentation of FluoZin-3 fluorescence is largely dependent on Zn^{2+} delivered from intracellular Zn^{2+} source(s).

Change in FluoZin-3 fluorescence by $ZnCl_2$

Zinc has been reported to exert protective action on the cells suffering from oxidative stress [3, 41–43]. To see if externally-applied zinc increases intracellular Zn^{2+} concentration, the effect of $ZnCl_2$ on FluoZin-3 fluorescence was examined. The concentration of $ZnCl_2$ was ranging from 0.3 to 30 μM because of physiological and pharmacological zinc concentrations reported [44–47]. As shown in Fig. 4a, the incubation with 10 or 30 μM $ZnCl_2$ for 90 min shifted the histogram of FluoZin-3 fluorescence to a direction of higher intensity, indicating $ZnCl_2$-induced increase in intracellular Zn^{2+} concentration. $ZnCl_2$ at concentrations of 1–30 μM significantly augmented FluoZin-3 fluorescence



(A)



(B)

Fig. 4 Change in FluoZin-3 fluorescence intensity by $ZnCl_2$. **a** $ZnCl_2$-induced shift of FluoZin-3 fluorescence histogram. FluoZin-3 fluorescence was measured from cells that were not stained with propidium iodide. Each histogram was constructed with 2,500 cells. **b** Concentration-dependent augmentation of FluoZin-3 fluorescence by $ZnCl_2$. *Columns* and *bars* indicate means and standard deviations, respectively, of five experiments. $^{#}P < 0.05$, $^{##}P < 0.01$ significant differences compared with control

in a concentration-dependent manner (Fig. 4b). Results suggest that externally-applied $ZnCl_2$ concentration-dependently increases intracellular Zn^{2+} concentration and that Zn^{2+} passes across membranes into the cells. The Zn^{2+} influx in this preparation was completely attenuated under cold temperature (3–4°C) [48]. Therefore, it may be dependent on metabolic process.

Change in 5-CMF fluorescence by $ZnCl_2$

In order to reveal the effect of $ZnCl_2$ on cellular thiol content, the effect on 5-CMF fluorescence was examined. As shown in Fig. 5a, the incubation with 10 or 30 μM $ZnCl_2$ for 90 min shifted the histogram of 5-CMF fluorescence to a direction of higher intensity, indicating $ZnCl_2$-induced increase in cellular content of nonprotein thiol, presumably glutathione [30]. $ZnCl_2$ at 10 and 30 μM significantly augmented 5-CMF fluorescence when the cells were incubated with $ZnCl_2$ for 90 min (Fig. 5b),

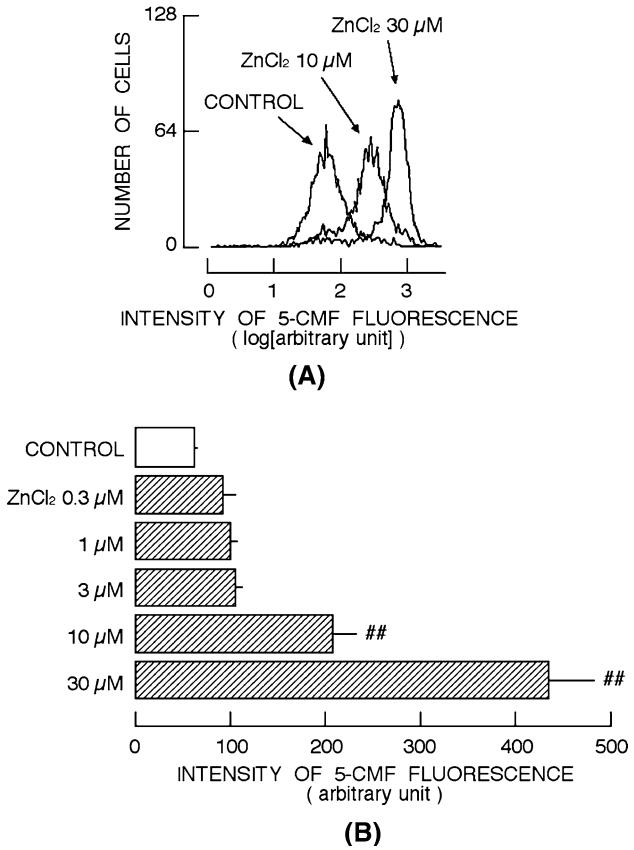


Fig. 5 Change in 5-CMF fluorescence intensity by $ZnCl_2$. **a** $ZnCl_2$-induced shift of 5-CMF fluorescence histogram. 5-CMF fluorescence was measured from the cells that were not stained with propidium iodide. Each histogram was constructed with 2,500 cells. **b** Concentration-dependent augmentation of 5-CMF fluorescence by $ZnCl_2$. *Columns* and *bars* indicate means and standard deviations, respectively, of five experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significant differences compared with control

indicating that $ZnCl_2$ concentration-dependently increases cellular thiol content.

Correlation between $ZnCl_2$-induced changes in 5-CMF and FluoZin-3 fluorescence

Since the incubation with $ZnCl_2$ augmented both 5-CMF and FluoZin-3 fluorescences (Figs. 4, 5), the correlation between them was examined. As shown in Fig. 6, there was 0.9952 for correlation coefficient when the cells were incubated without and with $ZnCl_2$ (0.3–30 μM).

Discussion

Effect of NEM

N-ethylmaleimide is widely used to decrease (or deplete) the cellular content of nonprotein thiols, mainly glutathione

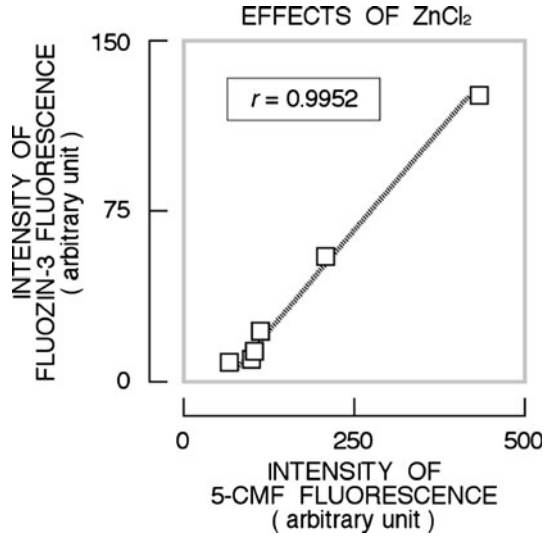


Fig. 6 Correlation between $ZnCl_2$-induced changes in FluoZin-3 and 5-CMF fluorescences. Each *point* was obtained from the results shown in Figs. 5 and 6 at respective concentrations of $ZnCl_2$

because it forms covalent bonds with sulfhydryl groups. Therefore, the use of NEM is suitable to make quantitative simulations of oxidative stress. As shown in Fig. 1, NEM concentration-dependently decreased the intensity of 5-CMF fluorescence that reflects the cellular content of nonprotein thiols, mainly glutathione [30]. The results indicate that NEM decreased cellular thiol content. In contrast, NEM increased the intensity of FluoZin-3 fluorescence (Fig. 2), an indicator for intracellular Zn^{2+} concentration [36]. The augmentation of FluoZin-3 fluorescence by NEM was not practically affected by the chelation of extracellular Zn^{2+} by DTPA (Fig. 3). Taken together, it is likely that NEM increases intracellular Zn^{2+} concentration by releasing Zn^{2+} from intracellular source(s). The changes in 5-CMF and FluoZin-3 fluorescences by NEM suggest that the decrease in cellular content of nonprotein thiols by NEM is associated with the increase in intracellular Zn^{2+} concentration (Figs. 1, 2 and 3). Such a negative relationship between them is true because intracellular Zn^{2+} makes a complex with the thiol group of protein and nonprotein [17–19], and the modification from thiol to disulfide releases Zn^{2+} from protein and nonprotein [20, 21]. NEM forms covalent bonds with sulfhydryl groups in thiols, resulting in Zn^{2+} release when Zn^{2+} makes a complex with thiol.

Effect of $ZnCl_2$

Zinc itself exerts an antioxidative action, leading to a protective action on the cells suffering from oxidative stress [3, 42, 43, 45]. As shown in Fig. 4, $ZnCl_2$ increased the intensity of FluoZin-3 fluorescence, indicating a $ZnCl_2$-induced increase in intracellular Zn^{2+} concentration. This

result indicates that Zn^{2+} passes across membranes, resulting in an increase in intracellular Zn^{2+} concentration. Since this membrane Zn^{2+} transport is completely blocked at a temperature of 4°C [48], it is not passive Zn^{2+} transport along electrochemical gradient of Zn^{2+}. $ZnCl_2$ also increased the intensity of 5-CMF fluorescence (Fig. 5), indicating an increase in cellular thiol content. The changes in FluoZin-3 and 5-CMF fluorescences by $ZnCl_2$ show a positive correlation with a correlation coefficient of 0.9952 (Fig. 6). Thus, taken together, the results indicate that the increase in intracellular Zn^{2+} concentration leads to the increase in cellular thiol content.

Implication

It is presumable that the cells have an ability to restore the cellular content of nonprotein thiols against oxidative stress that decreases the content. However, the trigger to restore the cellular content of nonprotein thiols during oxidative stress has not been elucidated. Oxidative stress is induced by reactive oxygen species such as hydrogen peroxide, superoxide anion, hydroxyl radical, and nitric oxide. Hydrogen peroxide decreases the cellular content of nonprotein thiols and increases the intracellular Zn^{2+} concentration by releasing Zn^{2+} from intracellular source(s) [15]; NOR-3, a donor of nitric oxide, also does so [49]. NEM is suggested to decrease the cellular thiol content and increase the intracellular Zn^{2+} concentration from the results of Figs. 1 and 2. Therefore, intracellular Zn^{2+} may be a trigger to restore the cellular thiol content. Zinc exerts an antioxidative action by increasing the cellular content of glutathione [7, 8]. In fact, $ZnCl_2$ correlatively increased the intensities of FluoZin-3 and 5-CMF fluorescences in a concentration-dependent manner (Figs. 4, 5 and 6). Therefore, it is hypothesized that intracellular Zn^{2+} released from intracellular stores during oxidative stress is a trigger to restore the cellular content of nonprotein thiols that is decreased by oxidative stress.

Zinc is reported to increase the glutathione level through the induction of a de novo synthesis pathway in ARPE-19 cells [7] or by increasing the transcription of the catalytic subunit of glutamate-cysteine ligase in primary rat endothelial cells [8]. Therefore, the increase in the intracellular Zn^{2+} concentration by oxidative stress may promote de novo synthesis of glutathione. Many chemicals that induce apoptosis in lymphocytes are either oxidants or activators of cellular oxidative metabolisms [50–53]. Therefore, the increase in cellular thiol content by Zn^{2+} released by oxidative stress has an important role in the protection of cells against oxidative stress.

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Conflict of interest None.

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