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Nitroprusside increases intracellular Zn^{2+} concentration without affecting cellular thiol content: A model experiment using rat thymocytes and FluoZin-3

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ABSTRACT

Nitric oxide (NO) is cytotoxic under some conditions although it has physiological roles. It is recently proposed that the cytotoxicity of NO is resulted from its interaction with glutathione and zinc. Since we have revealed that a decrease in cellular content of non-protein thiols, presumably glutathione, induces intracellular Zn^{2+} release, there is a possibility that the cytotoxicity of nitroprusside, a donor of NO, is resulted from the interaction of NO with cellular thiols, leading to an increase in intracellular Zn^{2+} concentration. To test the possibility, the effects of nitroprusside on cell lethality, intracellular thiol content, and intracellular Zn^{2+} concentration were examined in rat thymocytes by using a flow cytometer with propidium iodide and FluoZin-3. Nitroprusside at concentrations of 0.3 mM or more (up to 10 mM) significantly augmented FluoZin-3 fluorescence, indicating an increase in intracellular Zn^{2+} concentration. It was also the case under external Zn^{2+}-free condition, suggesting nitroprusside-induced release of intracellular Zn^{2+}. However, nitroprusside at 10 mM did not affect cell lethality and cellular thiol content. Thus, it can be concluded that nitroprusside-induced increase in intracellular Zn^{2+} concentration is not related to its cytotoxicity.

Keywords: zinc; nitroprusside; cytotoxicity; nitric oxide

1. INTRODUCTION

It is proposed that chemicals decreasing cellular thiol content increase intracellular Zn^{2+} concentration (Hashimoto *et al*., 2009; Kawanai *et al*., 2009; Oyama *et al*., 2009). This proposal is based on good correlation between the augmentation of FluoZin-3 fluorescence, an indicator for intracellular Zn^{2+}, and the attenuation of 5-chloromethylfluorescein (5CMF) fluorescence, an indicator for cellular nonprotein thiols when the cells are incubated with chemicals. Hydrogen peroxide, one of biological reactive oxygen species, also decreases the intensity of 5CMF fluorescence and increases that of FluoZin-3 fluorescence (Hashimoto *et al*., 2009; Oyama *et al*., 2009). Nitric oxide (NO) is one of free radical species and it reacts with superoxide anion to generate peroxynitrite that is proposed to induce Zn^{2+} release from intracellular stores (Bossy-Wetzel *et al*., 2004; St. Croix *et al*., 2005; Weissmann, 2008; Bernal *et al*., 2008). Nitroprusside is clinically used as a vasodilator by donating NO (Walker and Geniton, 1989). Therefore, there is a possibility that its cytotoxicity is resulted from the interaction of NO with cellular thiols, leading to an increase in intracellular Zn^{2+} concentration. To test the possibility, the effects of nitroprusside on cell lethality, intracellular thiol content, and intracellular Zn^{2+} concentration have been examined in rat thymocytes by using a flow cytometer with propidium iodide and FluoZin-3.

2. MATERIALS AND METHODS

2.1. Chemicals

Nitroprusside (NTRPS in Figs. 1-4) was purchased from Wako Pure Chemicals (Osaka, Japan). Diethylenetriamine- N,N,N',N",N"-pentaacetic acid (DTPA) was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). Fluorescent probes such as propidium iodide, FluoZin-3 pentaacetoxymethyl ester (FluoZin-3-AM), and 5-chloromethylfluorescein diacetate (5-CMF-DA) were the products of Molecular Probes Inc. (Eugene, Oregon, USA). NaCl, $CaCl_2$, $MgCl_2$, KCl, NaOH, $ZnCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glucose, and dimethyl sulfoxide (DMSO) were also purchased from Wako Pure Chemicals. Final concentration of DMSO as a solvent for FluoZin-3-AM in cell suspension was 0.1 % or less. The incubation with DMSO at 0.3 % or less did not affect the viability of rat thymocytes

during experiments.

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 (1-4ºC). 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 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 5×10^5 cells/ml). The beaker containing the cell suspension was water-bathed at 36ºC for 1 hr before the experiment.

2.3. Experimental protocol

Nitroprusside was added to cell suspension (2 ml cell suspension in 10 ml test tube). To examine the effect of DTPA, the agent was added to the suspension just before applying H_2O_2. The cell density was about 5×10^5 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 2×10^3 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.

FluoZin-3-AM (Gee *et al*., 2002) 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 (Hashimoto *et al*., 2009; Kawanai *et al*., 2009; Oyama *et al*., 2009). FluoZin-3 fluorescence was measured from the cells that were not stained with 5 μM propidium iodide (Matsui *et al*., 2008). 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 (Chikahisa et al., 1996). 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.

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. Effects of nitroprusside on cell lethality and cytogram of rat thymocytes

As shown in Fig. 1, the incubation of rat thymocytes with 10 mM nitroprusside for 3 hr did not increase the population of cells exerting propidium fluorescence. Thus, nitroprusside at 10 mM did not increase the population of dead cells and/or the cell with compromised membranes, indicating that the cell lethality was not changed by 10 mM nitroprusside.

Cell shrinkage is one of sensitive parameters for detecting cytotoxicity (Nakao *et al*., 2003; Iwase *et al*., 2004; Ogata *et al*., 2010). It occurs before chemicals induce cell death. Therefore, the effect of 10 mM nitroprusside on cytogram (forward scatter *versus* side scatter) was examined. As shown in Fig. 2, the incubation with 10 mM for 3 hr did not change the cytogram. Thus, nitroprusside at 10 mM did not exert cytotoxic action on rat thymocytes under present *in vitro* condition.

3.2. Effect of nitroprusside on intracellular Zn^{2+} concentration of rat thymocytes

The effect of nitroprusside at concentrations of 0.03-10 mM on the cells preloaded with FluoZin-3 was tested at 1-3 hr after the start of drug application. Nitroprusside at 10 mM time-dependently shifted the histogram of FluoZin-3 fluorescence monitored from the cells to a direction of higher intensity, indicating an increase in intracellular Zn^{2+} concentration. The shift attained a steady level within 1 hr after the start of drug application.

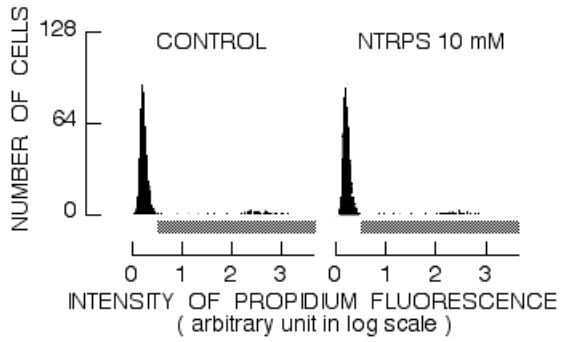

Fig.1. *Effect of nitroprusside (NTRPS) on cell lethality or rat thymocytes. Lethality was estimated from the population of cells stained with propidium iodide. Each histogram was constructed from* 2×10^3 *cells. Bar under the histogram indicates the region of cells exerting propidium fluorescence, presumably dead cells or the cells with compromised membranes.*

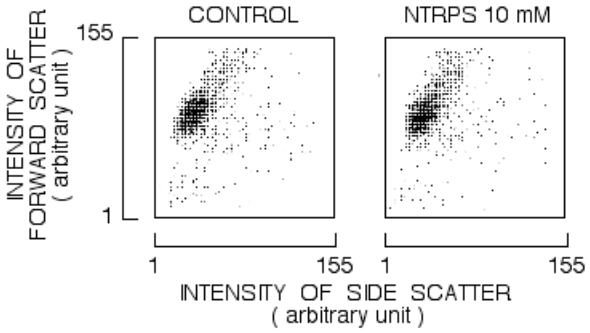

Fig. 2. *Effect of nitroprusside (NTRPS) on cytogram (forward scatter versus side scatter) of rat thymocytes. Each cytogram was constructed with* 2×10^3 *cells.*

As shown in upper panel of Fig. 3, the incubation with 1-10 mM nitroprusside shifted the histogram to a direction of higher intensity of FluoZin-3 fluorescence in a concentration-dependent manner. Thus, millimolar nitroprusside seems to increase intracellular Zn^{2+} concentration of almost all cells. Concentration-dependent increase in mean intensity of FluoZin-3 fluorescence is summarized in lower panel of Fig. 3. The threshold concentration of nitroprusside to increase intracellular Zn^{2+} concentration was 0.3 mM. The solution surrounding the cells contained Zn^{2+} derived from the cell preparation (Sakanashi *et al*., 2009) although the chemical composition of solution did not contain any zinc salts. Therefore, one may argue that nitroprusside-induced increase in intracellular Zn^{2+} concentration is due to the influx of extracellular Zn^{2+}. To confirm the source of Zn^{2+}, the effect of 0.03-10 mM nitroprusside was tested under external Ca^{2+} and Zn^{2+}-free condition where $CaCl_2$ was replaced with equimolar $MgCl_2$ and 10 μM DTPA, a chelator for extracellular Zn^{2+}, was added. Similar concentration-dependent augmentation of FluoZin-3 fluorescence by nitroprusside was observed under external Ca^{2+} and Zn^{2+}-free condition (not shown). This result indicates that nitroprusside-induced increase in intracellular Zn^{2+} concentration is due to intracellular Zn^{2+} release.

3.3. Effect of nitroprusside on cellular thiol content of rat thymocytes

Nitroprusside donates NO that induces oxidative stress to the cells, possibly resulting in release of Zn^{2+} from thiols as cellular Zn^{2+} binding sites. To test the possibility, the effect of millimolar nitroprusside on 5-CMF fluorescence of rat thymocytes was examined. As shown in left panel of Fig. 4, the incubation with 10 mM nitroprusside did not affect the histogram of 5-CMF fluorescence, indicating no effect of nitroprusside on cellular thiol content. Significant effect on cellular thiol content was not observed in all cases of 1-10 mM nitroprusside.

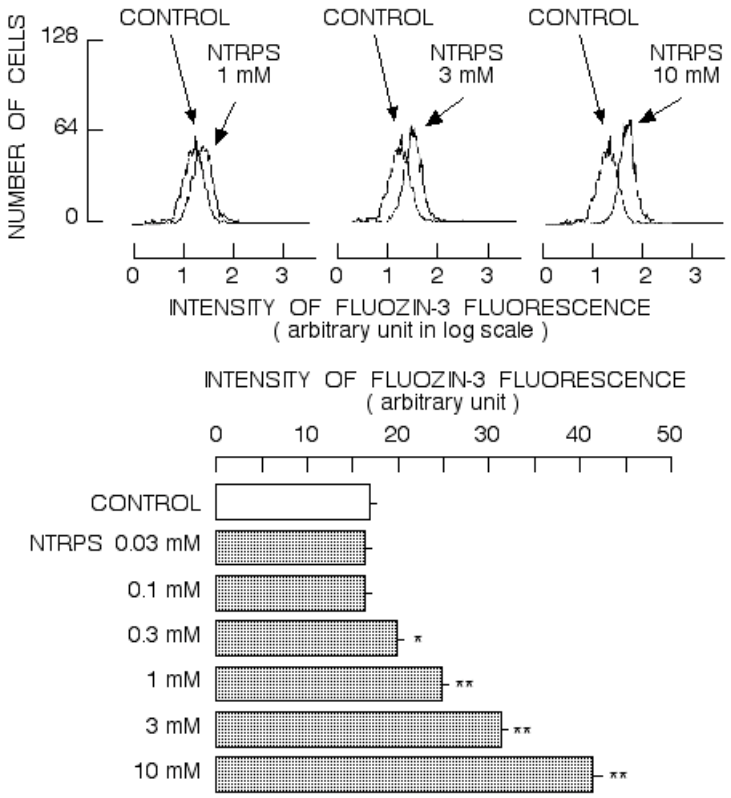

Fig. 3. *Effect of nitroprusside (NTRPS) on intracellular Zn^{2+} concentration. The change in intracellular Zn^{2+} concentration was estimated with FluoZin-3 fluorescence. Upper panel: Effect of NTRPS on the histogram of FluoZin-3 fluorescence monitored from 2×10^3 cells. Lower panel: Concentration-dependent effect of NTRPS on mean intensity of FluoZin-3 fluorescence. Column and bar respectively indicate mean and its standard deviation of four experiments. Symbols (*, **) indicate significant difference ($P < 0.05$, $P < 0.01$) to the control.*

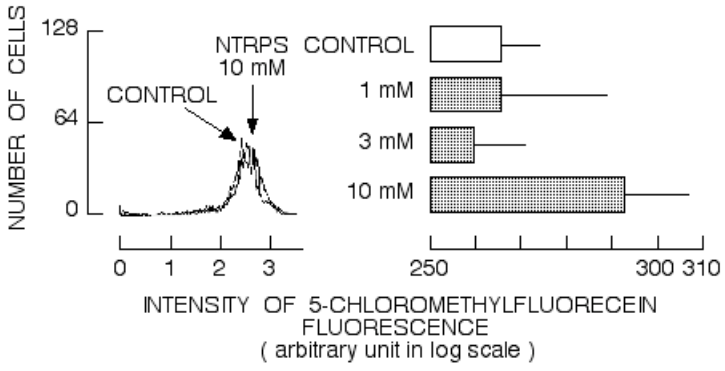

Fig. 4. *Effect of nitroprusside (NTRPS) on cellular content of nonprotein thiols. The change in cellular thiol content was estimated with 5-chlorofluorescein fluorescence. Left panel: Effect of NTRPS on the histogram of 5-chlorofluorescein fluorescence monitored from 2×10^3 cells. Right panel: Concentration-dependent effect of NTRPS on mean intensity of 5-chlorofluorescein fluorescence. Column and bar respectively indicate mean and its standard deviation of four experiments. Difference between them was not significant.*

4. DISCUSSION

The concentration of nitroprusside to increase intracellular Zn^{2+} concentration under *in vitro* condition was 0.3 mM or more (Fig. 3). Because these millimolar concentrations of nitroprusside seem to be higher than those observed under clinical conditions (Schulz, 1984; Friederich and Butterworth, 1995), it is quite difficult to suggest clinical implications on nitroprusside-induced increase in intracellular Zn^{2+} concentration. However, the study may reveal new pathway of drug-induced increase in intracellular Zn^{2+} concentration. Intracellular Zn^{2+} makes a complex with thiol group of protein and nonprotein (Jacob *et al*., 1998; Maret and Vallee, 1998; Gelinsky *et al*., 2002). The modification from thiol to disulfide releases Zn^{2+} from protein and nonprotein (Maret, 1994; Quesada *et al*., 1996). In fact, there is a good correlation between the augmentation of FluoZin-3 fluorescence, an indicator for intracellular Zn^{2+}, and the attenuation of 5-CMF fluorescence, an indicator for cellular nonprotein thiols when the cells are incubated with chemicals (Hashimoto *et al*., 2009; Oyama *et al*., 2009; Kawanai *et al*., 2009). However, nitroprusside did not decrease cellular thiol content in this study (Fig. 4). Thus, the mechanism for nitroprusside-induced increase in intracellular Zn^{2+} concentration cannot be explained by nitroprusside-induced oxidative stress （Bossy-Wetzel *et al*., 2004; St. Croix *et al*., 2005; Weissmann, 2008; Bernal *et al*., 2008）. Since nitroprusside releases not only NO but also cyanide and iron, cyanide and iron may be factors to induce intracellular Zn^{2+} release. In this aspect, further studies on cyanide and iron will be necessary.

In this study, nitroprusside even at 10 mM did not increase cell lethality (Fig. 1) and induce cell shrinkage (Fig. 2). Furthermore, the cellular thiol content was not affected by nitroprusside (Fig. 4). Therefore, the increase in intracellular Zn^{2+} concentration by nitroprusside is not related to the cytotoxicity of nitroprusside. It is unlikely that the cytotoxicity of nitroprusside, a donor of NO, is resulted from the interaction of NO with cellular thiols, leading to an increase in intracellular Zn^{2+} concentration.

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