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A comparison of the cellular actions of polaprezinc (zinc-L-carnosine) and $ZnCl_2$

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ABSTRACT

Aims: Zinc supplementation has been proven to be beneficial for the prevention of some health problems. Many zinc supplements are used for medical and nutritional purposes. However, it is difficult to distinguish between them in terms of their cellular actions. We compared the cellular actions of polaprezinc (zinc-L-carnosine) with those of $ZnCl_2$ in order to determine whether polaprezinc has greater zinc-related actions than $ZnCl_2$.
Main methods: Cellular actions of polaprezinc and $ZnCl_2$ were estimated by flow-cytometric techniques with appropriate fluorescent probes in rat thymocytes.
Key findings: Both agents had almost equal stimulatory effects on the intracellular Zn^{2+} level and cellular level of nonprotein thiol in a similar concentration-dependent manner. However, the increase in cell lethality caused by $ZnCl_2$ under severe oxidative stress was significantly greater than that caused by polaprezinc.
Significance: There are various zinc supplements, for example, zinc gluconate, zinc picolinate, and zinc methionine. However, the differences in their cellular actions have not been elucidated to date. Such studies could distinguish between zinc supplements.

Introduction

Zinc reduces the incidence, severity, and duration of diarrhea in children in developing countries (Fischer-Walker and Black, 2004; Hoque and Binder, 2006). Several studies have shown beneficial actions of zinc in the treatment of shigellosis via the modulation of immune functions (Raqib et al., 2004; Rahman et al., 2005). Zinc is important to T-cell maturation as it is a co-factor in the production of thymulin, a thymic hormone essential for T-cell production and function (Bach and Dardenne, 1989; Reggiani et al., 2009). It is likely that zinc is an effective adjunct therapeutic agent for the treatment of microbial infection (for a review see, Prasad, 2009).

Treatment with polaprezinc (zinc-L-carnosine), an antiulcer drug used in Japan, was also shown to improve *Helicobacter pylori* infection when it was administered together with an antimicrobial agent (Kashimura et al., 1999). In addition, zinc-supplying compounds such as polaprezinc are believed to suppress autoimmune disease by inhibiting T cell activation (Ohkawara et al., 2005; Tran et al., 2007). Polaprezinc has further been used in the treatment of hepatic fibrosis (Takahashi et al., 2007), acetaminophen-induced hepatitis (Nishida et al., 2010), and endotoxin shock (Ohata et al., 2010).

These actions, described above, of polaprezinc are linked to those of the zinc it contains. Therefore, one may ask if the actions of polaprezinc are different from those of a pure zinc salt such as $ZnCl_2$. In this cytometric study using appropriate fluorescent probes and rat thymocytes, we compared the cellular actions of polaprezinc with those of $ZnCl_2$ in order to determine whether polaprezinc has greater zinc-related actions than $ZnCl_2$.

Materials and methods

Chemicals

Polaprezinc, NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and H_2O_2 were obtained from Wako Pure Chemicals (Osaka, Japan). Distilled water was purchased from Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan). FluoZin-3-AM, 5-chloromethylfluorescein diacetate (5-CMF-DA), dihydroethidium, and propidium iodide were obtained from Molecular Probes Inc. (Invitrogen Corporation; Eugene, Oregon, USA).

Animals and cell preparation

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

The cell suspension preparation was similar to that previously reported (Chikahisa et al., 1996; Matsui et al., 2008). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 1–2 mm with a blade under cold conditions (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; pH adjusted to 7.3–7.4 with NaOH) to dissociate the thymocytes. Thereafter, the

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Tyrode's solution containing the cells was passed through a mesh (10 μm diameter) to prepare the cell suspension. The beaker containing the cell suspension was incubated in a water bath at 36–37 °C for 1 h before the experiment. Although the Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM zinc derived from the cell preparation (Sakanashi et al., 2009).

Rat thymocytes were used for the study because single cells can be prepared without enzymatic treatment, and thus, their cell membranes remain intact. In addition, the modification of cell death (apoptosis and necrosis) by zinc has been studied extensively in murine thymocytes (Barbieri et al., 1992; McCabe et al., 1993; Provinciali et al., 1995; Maclean et al., 2001).

Fluorescence measurements of cellular and membrane parameters

The methods for measuring the 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 described previously (Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed by JASCO software (Version 3.06; JASCO, Tokyo, Japan). There was no fluorescence from the reagents used in the study, except for the fluorescent probes, under our experimental conditions.

To assess cell lethality, propidium iodide was added to the cell suspensions to a final concentration of 5 μM. Because propidium stains dead cells, the measurement of propidium fluorescence from cells provided information on lethality. The fluorescence was measured 2 min after the application of propidium iodide by using a flow cytometer. The excitation wavelength used for propidium was 488 nm, and the emission was detected at 600 ± 20 nm.

FluoZin-3-AM (Gee et al., 2002) was used as an indicator of intracellular Zn^{2+}. The cells were incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements were taken to estimate the change in the intracellular Zn^{2+} concentrations of the rat thymocytes with intact membranes. FluoZin-3 fluorescence was measured in the cells that were not stained with 5 μM propidium iodide (Matsui et al., 2008). The excitation wavelength used for FluoZin-3 was 488 nm, and the emission was detected at 530 ± 20 nm.

5-CMF-DA was used to monitor changes in the 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 were taken. 5-CMF fluorescence was measured in the cells that were not stained with 5 μM propidium iodide. The excitation wavelength used for 5-CMF was 488 nm, and the emission was detected at 530 ± 15 nm.

Dihydroethidium was used to detect intracellular superoxide anions (Fink et al., 2004). The cells were incubated with 10 μM dihydroethidium for 60 min before any fluorescence measurements were taken. The excitation wavelength used was 488 nm, and the emission was detected at 600 ± 15 nm.

Protocol for cells subjected to oxidative stress

H_2O_2 (2 μL) was added to the cell suspension (2 mL). The cell density was approximately 5×10^5 cells/mL. To examine the effect of polaprezinc or $ZnCl_2$, the agent was added to the suspension just before applying H_2O_2. The cells were incubated with polaprezinc or $ZnCl_2$ and H_2O_2 at 36–37 °C. The data acquisition of fluorescence from 2×10^3 cells by using a flow cytometer required at least 10 s. The effect of H_2O_2 on the thymocytes was examined in a preliminary study. H_2O_2 at 2 mM was lethal to the thymocytes when the exposures lasted 60 min or longer. This was determined based on the prolonged exposure of thymocytes to 2 mM H_2O_2 increasing the number of cells stained with propidium iodide (presumably dead cells and/or cells with compromised membranes). The incubation of the cells with 2 mM H_2O_2 for 90–120 min induced a small increase (10–20%) in cell lethality that was suitable for examining the potentiation of H_2O_2-induced cytotoxicity by the agent. H_2O_2 at 100 μM was not lethal, but it increased the intensity of FluoZin-3 fluorescence (Matsui et al., 2008).

Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed with Tukey's multivariate analysis. A P value of <0.05 was considered significant.

Results

Effects of polaprezinc and $ZnCl_2$ on the intracellular Zn^{2+} concentration

Polaprezinc, at a concentration of 0.3 μM, caused a small increase in the intensity of the FluoZin-3 fluorescence (Fig. 1). As shown in Fig. 1, further increases in the concentration of polaprezinc (up to 10 μM) induced further increases in the intensity of the fluorescence, in a concentration-dependent manner. The potency of polaprezinc, in terms of its ability to augment FluoZin-3 fluorescence, was similar to that of $ZnCl_2$ when their effects were examined 90 min after the start of drug application.

Effects of polaprezinc and $ZnCl_2$ on the cellular content of nonprotein thiols

The incubation of the cells with polaprezinc at concentrations ranging from 0.3 μM to 10 μM increased the intensity of 5-CMF fluorescence in a concentration-dependent manner (Fig. 2). Significant increases in the intensity were observed when the concentration of polaprezinc was 1 μM or greater. Polaprezinc at concentrations of 1 μM or greater therefore appeared to increase the cellular content of nonprotein thiols. Similar to the findings for FluoZin-3 fluorescence, the potency of polaprezinc, in terms of its ability to augment 5-CMF fluorescence, was similar to that of $ZnCl_2$ when their actions were examined 90 min after the start of drug application.

Effects of polaprezinc and $ZnCl_2$ on the viability of cells treated with H_2O_2

Micromolar zinc has been shown to exert a cytotoxic action on cells experiencing oxidative stress induced by H_2O_2 (Matsui et al., 2009, Matsui et al., 2010). To observe if this was also the case for polaprezinc, the effect of polaprezinc on the cells simultaneously incubated with H_2O_2 was examined. The incubation of cells with 10 μM polaprezinc or 10 μM $ZnCl_2$ for 90 min did not affect cell lethality. Cell lethality was, however, increased by the incubation with H_2O_2 at 2 mM, and

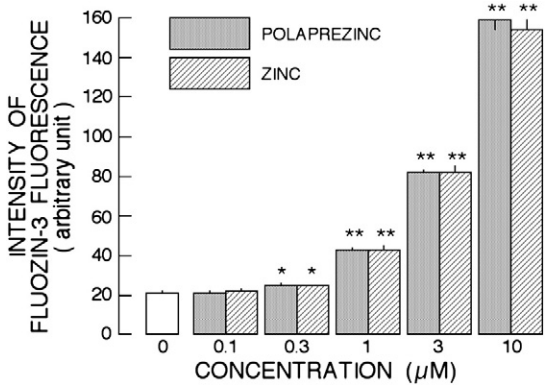

Fig. 1. The effects of polaprezinc and $ZnCl_2$ on the FluoZin-3 fluorescence of rat thymocytes. Effects were examined 60 min after the start of incubation with the respective agent. Columns and bars indicate the means and standard deviations of 4 or 5 experiments, respectively. The asterisks (*, **) indicate significant differences ($P<0.05$, $P<0.01$) between the control group and treated groups.