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Zinc-related actions of sublethal levels of benzalkonium chloride: Potentiation of benzalkonium cytotoxicity by zinc

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ABSTRACT

Benzalkonium chloride (BZK) is a common preservative used in pharmaceutical and personal care products. $ZnCl_2$ was recently reported to significantly potentiate the cytotoxicity of some biocidal compounds. In the present study, therefore, we compared the cytotoxic potency of BZK and then further studied the Zn^{2+}-related actions of the most cytotoxic agent among BZK, using flow cytometric techniques with appropriate fluorescent probes in rat thymocytes. Cytotoxicity of benzylcetyldimethylammonium (BZK-C16) was more potent that those of benzyldodecyldimethylammonium and benzyldimethyltetradecylammonium. $ZnCl_2$ (1–10 μM) significantly potentiated the cytotoxicity of BZK-C16 at a sublethal concentration (1 μM). The co-treatment of cells with 3 μM $ZnCl_2$ and 1 μM BZK-C16 increased the population of both living cells with phosphatidylserine exposed on membrane surfaces and dead cells. BZK-C16 at 0.3–1.0 μM elevated intracellular Zn^{2+} levels by increasing Zn^{2+} influx, and augmented the cytotoxicity of 100 μM H_2O_2. Zn^{2+} is concluded to facilitate the toxicity of BZK. We suggest that the toxicity of BZK is determined after taking extracellular (plasma) and/or environmental Zn^{2+} levels into account.

1. Introduction

Benzalkonium chloride (BZK) is a common preservative used in pharmaceutical and personal care products [37]. The concentration of BZK in such products is adjusted so that the minimum amount necessary to achieve antimicrobial actions is added. Some reports indicate an increased incidence of adverse effects after long-term use of products containing BZK (for a review, [16]. Additionally, BZK has been found to be genotoxic under some experimental conditions [1,3]. It may be impossible to recommend a safe limit for BZK in the general population because of conflicting data [16]. Recently, two inpatients were murdered with BZK that was mixed into their intravenous bags in Yokohama, Japan [35]. It is necessary to further study the cellular actions of BZK in order to accumulate basic information on its toxicity.

Zinc is involved in the cytotoxicity of some biocidal compounds. In brief, the chelator of intracellular Zn^{2+} greatly attenuates the increase in cell lethality induced by H_2O_2 [18], while $ZnCl_2$ augments the cytotoxicity of H_2O_2 [17]. Furthermore, $ZnCl_2$ potentiates the cytotoxicity of biocides such as 2-n-octyl-4-isothiazolin-3-one [4], 4,5-dichloro-2-octyl-4-isothiazolin-3-one [29], and 2,2-dibromo-3-nitrilopropionamide [9]. BZK is also one type of biocide. Therefore, we hypothesized that the cytotoxicity of BZK is potentiated by zinc, and evaluated the Zn^{2+}-related actions of BZK using flow cytometric techniques with appropriate fluorescent probes in rat thymocytes. Commercially available BZK consists of benzyldodecyldimethylammonium chloride, benzyldimethyltetradecylammonium chloride, and benzylcetyldimethylammonium chloride. In the present study, we compared their cytotoxic potency and then further studied the Zn^{2+}-related effect of the most potent agent among them.

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2. Materials and methods

2.1. Chemicals

Benzyldodecyldimethylammonium chloride dihydrate (BZK-C12, purity 99.7% estimated by HPLC), benzyldimethyltetradecylammonium chloride hydrate (BZK-C14, purity 98.9%), and benzylcetyldimethylammonium chloride hydrate (BZK-C16, purity 98.3%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Propidium iodide (PI), annexin V-FITC, FluoZin-3-AM, and 5-chloromethylfluorescein diacetate (5-CMF-DA) were obtained from Invitrogen (Eugene, OR, USA). WST assay kit and Zn^{2+} chelators (diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid and N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine; DTPA and TPEN, respectively) were obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Other chemicals were supplied from Wako Pure Chemicals unless mentioned.

2.2. Animals and cell preparation

This study (Registered number 05279) was approved by the Tokushima University Committee for Animal Experiments, Tokushima, Japan. The cell suspension was prepared as previously reported [2,17]. In brief, thymus glands were obtained from rats that were anesthetized with ether. The slices were gently triturated in cold Tyrode's solution (2–4 °C, 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.4 adjusted by 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and appropriate amount of NaOH) to dissociate single thymocytes. The solution containing dissociated cells was then passed through a mesh (56 μm in diameter) to prepare cell suspension. It is noted that the cell suspension contained 216.9 ± 14.4 nM zinc that was derived from cell preparation [30]. The cell suspension was incubated at 36–37 °C for 1 h before any experimentation. This preparation has experimental limitation. Thymocytes spontaneously undergo apoptosis during a prolonged incubation [5,21]. Therefore, the experiments were completed within 8 h after the dissection of thymus glands from rats.

Various concentrations of BZK (0.1–10 mM in distilled water) were added to the cell suspensions (2 mL per test tube) and thereafter incubated at 36–37 °C for 2–4 h. A sample from each cell suspension (100 μL) was analyzed by a flow cytometry to estimate the BZK-induced changes in cellular and membrane parameters. It took about 10 s to acquire data from 2500 cells.

2.3. Fluorescence measurements

Cellular and membrane parameters were measured using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes as previously described [2,19]. The fluorescence was analyzed by JASCO software in operating system (Version 3.06; JASCO, Tokyo, Japan). Under experimental conditions, no fluorescence from the reagents used was observed, except for the fluorescent probes. The excitation wavelength for all fluorescent proves used in this study was 488 nm, while emissions wavelengths were 530 ± 20 nm for FITC, FluoZin-3, and 5-CMF and 600 ± 20 nm for PI.

To assess cell lethality, PI was added to the cell suspensions to a final concentration of 5 μM. Because PI stains dead cells, the measurement of PI fluorescence provides information on cell lethality. The fluorescence was measured using a flow cytometer 2 min after adding PI. Exposure of phosphatidylserine on the outer surface of cell membranes, a phenomenon that occurs during the early stage of apoptosis, was detected using annexin V-FITC [13]. Cells were treated with annexin V-FITC (10 μl/mL) for 30 min before evaluation. FluoZin-3-AM was used to monitor changes in the intracellular Zn^{2+} levels ($[Zn^{2+}]_i$) [7]. The cells were treated with 1 μM FluoZin-3-AM for 60 min prior to any fluorescence measurements. 5-CMF-DA was used to estimate the cellular content of glutathione ($[GSH]_i$) in rat thymocytes [2]. Oxidative stress decreases $[GSH]_i$. The 5-CMF fluorescence was measured 30 min after adding 1 μM 5-CMF-DA because it attains peak intensity within 30 min after application. 5-CMF fluorescence was monitored in the living cells that were not stained with PI.

2.4. WST assay

Cells were incubated with the WST-1 reagent for 2 h in a 96-well tissue culture plate after the cells were treated with BZK-C16 at 0.3–10 μM for 2 h. Thereafter, the formation of formazan was quantitated with a microplate reader (MTP-310Lab, Corona Electric, Hitachinaka, Japan). The measured absorbance at 450 nm correlates with the number of viable cells.

2.5. Statistical analysis and presentation

Statistical analyses were done using ANOVA, with post-hoc Tukey's multivariate analysis. *P*-values of 0.05 or less were statistically considered significant. In the results, values (columns and bars in figures) were expressed as the mean and the standard deviation of 4–8 samples. Each experiment was repeated three times unless noted otherwise.

3. Results

3.1. Cytotoxic actions of BZK-C12, BZK-C14, and BZK-C16

As shown in Fig. 1A, the treatment of rat thymocytes with 3 μM BZK-C14 or BZK-C16 for 2 h increased the cells exhibiting PI fluorescence. However, as almost all cells showed PI fluorescence in the presence of BZK-C16, its cytotoxicity was considered more potent than that of BZK-C14. The intensity of forward scatter, a parameter of cell size, was decreased in a large population of cells with PI fluorescence, suggesting shrunken dead cells. Dose-dependent changes in cell lethality by BZK-C12, BZK-C14, and BZK-C16 are summarized in Fig. 1B. The potency order for cytotoxicity was BZK-C16 > BZK-C14 > BZK-C12 when the cells were treated with 3 μM of the respective agents for 2 h. The experiments described below were carried out using 0.1–1.0 μM of BZK-C16. The change in cell viability, estimated with WST assay, by BZK-C16 was shown in Fig. 1C. The treatment of cells with 0.1–3 μM BZK-C16 for 2 h did not decrease the absorbance caused by formazan dye while significant reduction of absorbance in the case of 3 μM BZK-C16. Result indicates the significant decrease in cell viability by 3 μM BZK-C16.

3.2. Effects of BZK-C16 on forward scatter and side scatter

The changes of cell lethality (Fig. 1B) and viability (Fig. 1C) by BZK-C16 were very steep. Therefore, the effects of BZK-C16 at sublethal concentrations (1 μM or less) on the cells were examined. The treatment of cells with 0.1–1 μM for 4 h did not significantly increase the number of cells exhibiting PI fluorescence (dead cells). However, BZK at 0.1–1 μM slightly, but significantly, decreased the intensity of side scatter without affecting forward scatter (Fig. 2). Both forward and side scatter were decreased by 1 μM BZK.

3.3. Potentiation of BZK-C16 cytotoxicity by $ZnCl_2$

Zinc is involved in the cytotoxicity of some chemical compounds [4,17,19,29]. To determine whether the cytotoxicity of BZK-C16 is