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Cetylpyridinium chloride at sublethal levels increases the susceptibility of rat thymic lymphocytes to oxidative stress

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HIGHLIGHTS

- Cetylpyridinium chloride (CPC) is an antimicrobial agent used in personal care products.
- CPC is subsequently released into the environment.
- Sublethal levels of CPC modified cellular parameters in rat thymocytes.
- CPC exerted profound cytotoxic action on the cells under oxidative stress.
- Environmentally-relevant levels of CPC is suggested to be bioactive under certain conditions.

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ABSTRACT

Cetylpyridinium chloride (CPC) is an antimicrobial agent used in many personal care products, with subsequent release into the environment. Since CPC is found at low concentrations in river and municipal wastewater, its influence on wildlife is of concern. Therefore, in this study, we used flow cytometry to examine the effects of sublethal concentrations of CPC on rat thymic lymphocytes in order to characterize the cellular actions of CPC at low concentrations in the presence and absence of H_2O_2-induced oxidative stress. CPC treatment increased the population of living cells with phosphatidylserine exposed on the outer surface of their plasma membranes (a marker of early stage apoptosis), elevated intracellular Zn^{2+} levels, and decreased the cellular content of nonprotein thiols. CPC also potentiated the cytotoxicity of H_2O_2. Our results suggest that, even at environmentally relevant sublethal concentrations, CPC exerts cytotoxic effects under oxidative stress conditions by increasing intracellular Zn^{2+} concentration and decreasing the cellular content of nonprotein thiols. These findings indicate that, under some in vitro conditions, CPC is bioactive at environmentally relevant concentrations. Therefore, CPC release from personal care products into the environment may need to be regulated to avoid its adverse effects on wildlife.

1. Introduction

Cetylpyridinium chloride (CPC), a quaternary ammonium compound belonging to the group of cationic antimicrobial agents, is used in various types of mouthwashes, toothpastes, and other personal care products such as lozenges, throat sprays, breath sprays, and nasal sprays. It is considered a safe antimicrobial agent with broad-spectrum activity for preventing biofilm formation and gingivitis (Watanabe et al., 2008). CPC is also used as a disinfectant in private and public areas. These include swimming pools and aquariums; facilities storing water used for bathing and other purposes; air-conditioning systems; walls and floors in healthcare and other institutions; chemical toilets; and in organizations dealing with waste water, hospital waste, soil, or other similar substrates (EC Scientific Committee on Consumer Safety, 2015).

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The reported toxicities of CPC under in vivo conditions were reviewed in the Revision of the Opinion on Cetylpyridinium Chloride (EC Scientific Committee on Consumer Safety, 2015). The use of CPC for oral or dermal applications is considered relatively safe. However, because CPC is used for the diverse purposes described above, a considerable amount is released into the environment. The CPC concentration in the river and municipal wastewater in Kaohsiung City, which is located in Southern Taiwan and has a population of approximately 2.77 million, was reported to be 52 μg/l and 47–88 μg/l, respectively (Shrivas and Wu, 2007). Therefore, the calculated molar concentration of CPC is 138–259 nM. CPC exhibited cytotoxic effects in human keratinocyte and murine fibroblast cell lines at concentrations of 0.0004–0.5% (11.7 μM–14.7 mM) (Fromm-Dornieden et al., 2015) and developmental and acute toxicity in frog embryos at 0.5–3 μM (Park et al., 2016). In addition, low CPC concentrations, ranging from 3 nM to 30 nM, have been shown to inhibit nuclear factor-κB-induced osteoclast formation in mouse bone marrow cells in a concentration-dependent manner (Zheng et al., 2013). Thus, it is likely that CPC exerts some cellular actions even at sublethal environmentally relevant concentrations. This raises concerns regarding the effects of low concentrations of CPC on wildlife. Therefore, in this study, we used flow cytometry to explore the cellular effects of CPC at sublethal concentrations in rat thymic lymphocytes.

2. Materials and methods

2.1. Reagents

CPC was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). FluoZin-3-AM, 5-chloromethylfluorescein diacetate (5-CMF-DA), annexin V-FITC, and propidium iodide were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Zn^{2+} chelators, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). DTPA chelates extracellular Zn^{2+} but not intracellular Zn^{2+} because the agent is not membrane-permeable. TPEN is a membrane-permeable Zn^{2+} chelator. TPEN chelates intracellular Zn^{2+}. Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise mentioned.

2.2. Cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). The cell suspension was prepared as previously reported (Chikahisa et al., 1996; Sakanashi et al., 2009; Matsui et al., 2010). In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions. The slices were triturated in Tyrode's solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES at pH 7.3–7.4 adjusted with a small amount of NaOH) to dissociate the thymocytes. The cell suspension was incubated at 36–37 °C for 1 h before the experiment. Importantly, the zinc concentration in the cell suspension was 216.9 ± 14.4 nM (Sakanashi et al., 2009). The cell suspension contained trace amounts of zinc derived from the cell preparation.

There was one technical limitation in present study. Thymocytes are the cells that spontaneously undergo apoptosis during the incubation for 24 h (Rinner et al., 1996; Nishimura et al., 2008). The incubation of rat thymocytes for 12 h or longer (up to 24 h) significantly increased the population of shrunken cells, one of parameters during early phase of apoptosis. Therefore, the experiments were performed within 6 h after the start of incubation.

2.3. Fluorescence measurements of cellular parameters

The methods employed 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 (Chikahisa et al., 1996; Matsui et al., 2010). The fluorescence was analyzed using JASCO software (JASCO). Excitation wavelength for fluorescent probes was 488 nm. FITC, FluoZin-3, and 5-CMF fluorescence were detected at 530 ± 20 nm. Propidium fluorescence was detected at 600 ± 20 nm.

To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells (or the cells with compromised membranes), the measurement of propidium fluorescence from cells provides a clue to estimate the lethality. The fluorescence was measured at 5 min after the application of propidium iodide by a flow cytometer.

To detect phosphatidylserine, annexin V-FITC was used in a combination with propidium iodide. Annexin V interacts strongly and specifically with phosphatidylserine and can be used to detect apoptosis by targeting for the loss of plasma membrane asymmetry. Phosphatidylserine is normally exposed on inner side of plasma membranes and it becomes exposed on outer membrane surface of the cells undergoing apoptosis. Therefore, annexin V-FITC is able to detect the surface changes in membrane surface that occur early during apoptosis (Koopman et al., 1994). Annexin V-FITC (10 μl/ml) and propidium iodide (5 μM), respectively, were added to cell suspension at 30 and 2 min before the measurement.

FluoZin-3 is a Zn^{2+}-selective indicator and exhibits high Zn^{2+}-binding affinity that is unperturbed by Ca^{2+} concentrations up to at least 1 μM (Gee et al., 2002). To estimate the change in intracellular Zn^{2+} levels of rat thymocytes with intact membranes, FluoZin-3-AM and propidium iodide were simultaneously used. Both dyes were added to the solution to achieve final concentrations of 500 nM for FluoZin-3-AM and 5 μM for propidium iodide. Cells were incubated with FluoZin-3-AM for 50–60 min before the application of CPC. FluoZin-3 fluorescence was measured from the cells that were not stained with propidium iodide (living cells with intact membranes).

5-CMF-DA was used to estimate cellular content of glutathione. The correlation coefficient between 5-CMF fluorescence intensity and cellular glutathione content was 0.965 in rat thymocytes (Chikahisa et al., 1996). Oxidative stress decreases cellular content of glutathione. The CMF fluorescence was measured at 30 min after the application of 1 μM CMF-DA because it attains peak intensity within 30 min after the application. 5-CMF fluorescence was

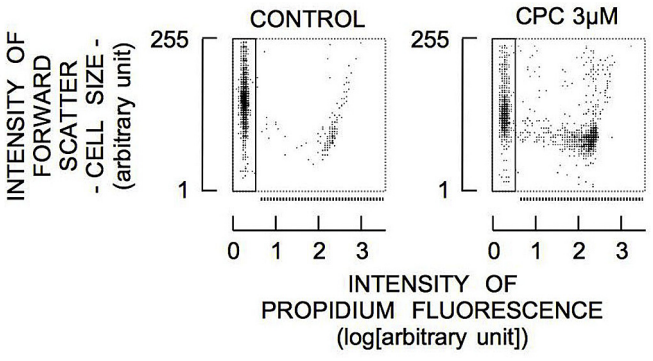

Fig. 1. Change in the cytogram (propidium fluorescence versus forward scatter) after 3 μM CPC treatment. The cytogram was obtained from 2500 cells. The dotted line under the cytogram indicates the population of cells exhibiting propidium fluorescence, dead cells, and/or the cells with compromised membranes.