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Triclocarban-induced change in intracellular Ca^{2+} level in rat thymocytes: Cytometric analysis with Fluo-3 under Zn^{2+}-free conditions

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ABSTRACT

Triclocarban (TCC) is an antimicrobial used in personal hygiene products. Recent health concerns arose after TCC was detected in the blood of human subjects who showered with soap containing TCC. In this study, the effect of TCC on intracellular Ca^{2+} concentration in rat thymocytes was examined using Fluo-3, an indicator of intracellular Ca^{2+}. TCC at concentrations ranging from 0.1 μM to 3 μM increased intracellular Ca^{2+} concentration biphasically: first by releasing Ca^{2+} from intracellular Ca^{2+} stores and then inducing Ca^{2+} influx through store-operated Ca^{2+} channels. The threshold TCC concentration to increase intracellular Ca^{2+} concentration in this study was lower than the maximum TCC concentrations reported in human blood samples. Therefore, we anticipate that TCC at concentrations reported in human blood samples might disturb intracellular Ca^{2+} signaling in human lymphocytes.

1. Introduction

Triclocarban (TCC) is used as an antimicrobial in personal hygiene products (Scientific Commitee on Consumer Products, 2005). This antimicrobial was found in waters of urban rivers around the world (Coogan and La Point, 2008; Zhao et al., 2010; Snyder et al., 2011; Schebb et al., 2011). Furthermore, TCC was detected in blood sampled from human subjects after showering with soap containing TCC (Schebb et al., 2012), with maximum blood concentrations ranging between 0.023 μM and 0.53 μM. Therefore, there are rising concerns that TCC might induce adverse cellular actions in humans and wild animals.

TCC at concentrations ranging from 0.05 μM to 0.5 μM significantly increases intracellular Zn^{2+} concentration in rat thymocytes and decreases the cellular content of nonprotein thiols (Morita et al., 2012). It is possible that TCC also affects intracellular Ca^{2+} concentration since some chemicals such as tributyltin (Chikahisa and Oyama, 1992; Oyama et al., 2009), methylmercury (Oyama et al., 1995; Kawanai et al., 2009) and thimerosal (Gukovskaya et al., 1992; Hashimoto

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et al., 2009) increase both Zn^{2+} and Ca^{2+} concentrations in murine lymphocytes. The increase in intracellular Ca^{2+} concentration is an essential triggering signal for lymphocyte activation by antigens and other stimuli (Lewis, 2001; Hogan et al., 2010). Therefore, from a toxicology point of view, it is interesting to know if TCC affects intracellular Ca^{2+} concentration. However, Zn^{2+} may disturb the measurement of intracellular Ca^{2+} concentration using chelator-based Ca^{2+} indicators such as Fluo-3 and Fura-2. For example, Zn^{2+} is bound to Fluo-3, a BAPTA-based Ca^{2+} indicator, in the presence of Ca^{2+} (Kao et al., 1989), and the stability constant of Zn^{2+}–BAPTA ($\log K = 9.38$) is higher than that of Ca^{2+}–BAPTA ($\log K = 6.97$). However, TCC-induced increase of FluoZin-3 fluorescence, a Zn^{2+} indicator (Gee et al., 2002), is completely inhibited by the use of *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a chelator of intracellular Zn^{2+} (Morita et al., 2012). Morita et al. (2012) reported that incubation of cells with triclocarban for 60 min significantly attenuated the fluorescence of 5-chloromethylfluorescein, a parameter of cellular thiol content. The TCC-induced changes in FluoZin-3 and 5-chloromethylfluorescein fluorescence had a correlation coefficient of −0.9225. Intracellular Zn^{2+} forms a complex with the thiol group of proteins and non-proteins (Jacob et al., 1998), and a modification from thiol to disulfide releases Zn^{2+} (Maret, 1994). One may argue the possibility that the an increase in the intracellular Zn^{2+} concentration by triclocarban affects the measurement of intracellular Ca^{2+} levels by fluorescent probes for Ca^{2+}. Use of *N,N,N′,N′*-tetra(2-picolyl)ethylenediamine (TPEN), a membrane permeable chelator of Zn^{2+}, is expected to be a tool to minimize the interference by Zn^{2+} in the measurement of Ca^{2+}. Minta et al. (1989) reported that Zn^{2+} binds to fluo-3 approximately 300-fold more strongly than Ca^{2+} does, and it increases the fluorescence of fluo-3.

In the present study, we examined TCC-induced changes of intracellular Ca^{2+} concentration in the presence of an appropriate concentration of TPEN by conventional flow cytometry techniques with Fluo-3. This paper describes the possible mechanism of such TCC-induced changes. This study has an implication on the toxicity of TCC, because the change in the intracellular concentration of free Ca^{2+} is an essential signal that triggers lymphocyte activation by an antigen and other stimuli to optimize T cell contact with the antigen-presenting cells and to increase the efficiency and specificity of transcription in B and T cells (Lewis, 2001). If TCC changes the intracellular Ca^{2+} concentration of lymphocytes, it would modify the immune functions.

2. Materials and methods

2.1. Chemicals

TCC, NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, and NaOH were obtained from Wako Pure Chemicals (Osaka, Japan). The chelator of intracellular Zn^{2+}, TPEN, was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). FluoZin-3-AM, Fluo-3-AM, propidium iodide, and annexin V-FITC were obtained from Molecular Probes Inc. Invitrogen (Eugene, OR, USA). TCC was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO as the solvent for TCC was 0.1%. In addition, Fluo-3-AM and FluoZin-3-AM were dissolved in DMSO. The application of Fluo-3-AM or FluoZin-3-AM increased the concentration of DMSO by 0.05%. DMSO at a concentration of 0.3% did not affect the viability, and the increase in the intensity of fluo-3 fluorescence by TCC with 0.1% DMSO was similar to that with 0.2% DMSO.

2.2. Animals and cell preparation

This *in vitro* study using thymocytes obtained from experimental animals was approved by the Committee for Animal Experiments at the University of Tokushima (Registered No. 05279). We used rat thymocytes in the present study, because the cell membranes of thymocytes remain intact and single cells can be prepared without enzymatic treatment and the Ca^{2+}-dependent process of cell death has been previously studied in murine thymocytes (Cohen and Duke, 1984; Kotelevskaia et al., 1991; Azmi et al., 1996).

We killed 21 male rats (6–8 weeks old) in this study. The cell suspension was prepared as 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 (NaCl, 150 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 5 mM; and HEPES, 5 mM; pH adjusted to 7.3–7.4 with NaOH) to dissociate the thymocytes. Thereafter, Tyrode's solution containing the cells was passed through a mesh (diameter, 10 μm) to prepare the cell suspension. The cell suspension was prepared within 15 min after the isolation of the thymus. The beaker containing the cell suspension was incubated in a water bath at 36–37 °C for 1 h before the experiment. Although Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM Zn^{2+}derived from the cell preparation (Sakanashi et al., 2009).

2.3. Fluorescence measurements of cellular and membrane parameters

The methods for measuring 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., 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 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 to estimate the change in the intracellular Zn^{2+}