— Research Letter —

Elevation of Intracellular Ca^{2+} Level by Triclosan in Rat Thymic Lymphocytes: Increase in Membrane Ca^{2+} Permeability and Induction of Intracellular Ca^{2+} Release

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Triclosan is an antibacterial agent used in household items and personal care products. Because wild animals and humans can harbor this compound in their systems, the toxic effects of triclosan are a possibility and are suspected. Therefore, we examined the effects of triclosan on intracellular Ca^{2+} concentration in rat thymocytes by cytometric techniques using fluorescent probes. Triclosan doses of 1–10 μM significantly increased the intensity of Ca^{2+}-detecting Fluo-3 fluorescence, indicating an increase in intracellular Ca^{2+} concentrations. The augmentation of Fluo-3 fluorescence became more profound in a dose-dependent manner after the addition of an external source of Ca^{2+}. Conversely, the removal of external Ca^{2+} greatly attenuated the triclosan-induced augmentation of Fluo-3 fluorescence. These results suggest that triclosan treatment allows external Ca^{2+} to pass through cell membranes. This phenomenon was not specific for Ca^{2+} because external Mn^{2+} quenched the triclosan-induced augmentation of Fluo-3 fluorescence, indicating that triclosan can also mediate Mn^{2+} permeation across membranes. Therefore, these results suggest that triclosan increases membrane permeability to divalent metal cations. Furthermore, triclosan induces Ca^{2+} release from intracellular stores because the Fluo-3 fluorescence intensity still increased slightly after triclosan treatment, even under conditions free from external Ca^{2+}. Additionally, triclosan did not increase the intensity of Fluo-3 fluorescence when Ca^{2+} was depleted from intracellular Ca^{2+} stores by A23187 under the external Ca^{2+}-free condition. Taken together, these data suggest that micromolar concentrations of triclosan affect intracellular Ca^{2+} homeostasis in thymocytes, possibly resulting in cellular malfunction.

Key words —— triclosan, Ca^{2+}, Fluo-3, thymocyte

INTRODUCTION

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) is used as an antibacterial agent in household items and personal care products.[1–3] Since triclosan-containing health care products are disposed of in residential drains,[4,5] triclosan is often detected in terrestrial and aquatic environments.[6–8] This agent is also found in the bodies of wild animals as well as humans,[9,10] including maternal milk[3,11] and blood.[12] Triclosan concentrations in blood plasma of human mothers were in the range of 0.4–38 μg/l.[3] Because human plasma concentrations of triclosan seem to be submicromolar (< 0.131 μM), it is unlikely that it affects the intracellular Ca^{2+} concentration of the cells present in the bloodstream. However, triclosan is a lipophilic compound (log K_{ow} = 4.8). Indeed, the concentrations of triclosan in lipophilic biological substances, including mammalian maternal milk and the bile of wild fishes, were measured at 60–300 μg/kg lipid weight (0.207–1.036 μmol/kg lipid weight) and 0.24–0.9 mg/kg fresh weight (0.828–3.108 μmol/kg fresh weight), respectively.[11] Therefore, the adverse effects of triclosan on these substances or on the cells contained in these substances are a topic of concern and are currently unclear.

The previously published *in vitro* experiments on triclosan toxicity were carried out with human gingival cells,[13,14] breast cancer cells,[15] natural killer cells,[16] and murine skeletal myotubes.[17] These studies found that micromolar triclosan concentrations induce various effects on the cells. Studies on murine myotubes showed that triclosan affected intracellular Ca^{2+} homeostasis such that it elevates intracellular Ca^{2+} level by releasing Ca^{2+} from the sarcoplasmic reticulum.[17] Since Ca^{2+} is

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recognized to have many physiological roles as a second messenger,[18, 19] the disruption of intracellular Ca^{2+} homeostasis by chemical compounds is assumed to lead to cytotoxicity. In an epidemiological study, higher levels of triclosan in young people correlated with increased diagnosis of allergies or hay fever, indicating that overexposure to triclosan may influence the human immune system.[20] Therefore, it is of interest to study the effect of triclosan on intracellular Ca^{2+} homeostasis of mammalian lymphocytes. In this study, we examined the characteristics of triclosan-induced increase in intracellular Ca^{2+} concentration in rat thymocytes (containing thymic lymphocytes) as measured by flow cytometric detection of fluorescent probes specific for cell death and Ca^{2+} flux, propidium iodide, and Fluo-3-pentaacetoxymethyl ester (Fluo-3-AM). Rat thymocytes were used in this study for the following 2 reasons. First, thymocyte cell membranes remain intact throughout the course of preparation because a single cell suspension can be prepared without an enzymatic treatment that might cause weakening or permeabilization of the cell membrane. This is important for this study because an intact cell membrane is necessary to study the effects of chemical treatment on intracellular Ca^{2+} concentrations due to a large transmembrane Ca^{2+} gradient. Second, the process of cell death is extensively studied in murine thymocytes; additionally, there are several types of hormones, biological compounds, and chemicals that have been identified to induce apoptosis and can be studied to determine whether they play a role in the functional outcome of triclosan-induced Ca^{2+} flux.[21,22]

MATERIALS AND METHODS

Chemicals —— Triclosan was purchased from Wako Pure Chemicals (Osaka, Japan). Fluo-3-AM and ethylendiaminetetraacetic acid (EDTA) were purchased from Dojin Chemical Laboratory (Kumamoto, Japan). Propidium iodide and A23187 were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Thapsigargin was obtained from Biomol International (Enzo Life Sciences Inc., Farmingdale, NY, U.S.A.). Other chemicals (NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, and NaOH) were purchased from Wako Pure Chemicals. The buffer for Tyrode's solution was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Nacalai Tesque, Kyoto, Japan).

Animals and Cell Preparation —— This study was approved by the Committee for Animal Experiments at the University of Tokushima (No. 05279). Male rats (Wistar strain, Charles River Laboratories Japan Inc., Yokohama, Japan) were provided with automatic water and a commercial diet (MF, Oriental Yeast, Tokyo, Japan) *ad libitum*. The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and it was artificially illuminated with fluorescent light on a 12-hr light/dark cycle (0800–2000). Cell suspension was prepared similar to that previously reported.[23, 24] In brief, thymus glands dissected from ether-anesthetized rats were sliced to 400–500 µm thickness with a razor under cold conditions (3–4°C). The slices were triturated by gently shaking them in chilled Ca^{2+}-free Tyrode's solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES adjusted to a pH of 7.3–7.4) to dissociate thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a 10-µm mesh to prepare a single-cell cell suspension. The cell suspension was maintained at 36–37°C in a water bath 1 hr before the experiment.

Fluorescence Measurements of Cellular and Membrane Parameters —— The methods for measurements of cellular and membrane parameters by using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described.[23, 24] The fluorescence was analyzed using the JASCO software (Ver. 3.06).

Fluo-3-AM was used as a fluorescent indicator for intracellular Ca^{2+}.[25] Rat thymocytes with intact membranes were incubated with 500 nM Fluo-3-AM for 60 min. After incubation, fluorescence measurements were made to estimate the change in intracellular Ca^{2+} concentration. The normal Ca^{2+} condition was prepared by adding 2 mM $CaCl_2$ to the cell suspension. Adding 2 mM $MgCl_2$ and 1 mM EDTA to chelate the free Ca^{2+} ions in the cell suspension was used to create the external Ca^{2+}-free condition. Fluo-3 fluorescence was measured from the cells that were not stained with 5 µM propidium iodide.[24] Since propidium stains dead cells, measuring the propidium fluorescence from the experimental cells enables the monitoring of Fluo-3 fluorescence from cells with intact membranes that represent only the living cells. Cell viability was estimated from the population of living cells that were not stained with propidium iodide. The excitation wavelength for Fluo-3 and propidium was 488 nm.

Emission was detected at 530 ± 20 nm for Fluo-3 fluorescence and 600 ± 20 nm for propidium fluorescence.

Statistics —— Values were expressed as the mean ± standard deviation of 3 experiments unless otherwise mentioned. Statistical analysis was performed with Tukey multivariate analysis. A p value of less than 0.05 was considered significant.

RESULTS

Time- and Concentration-dependent Changes in the Intensity of Fluo-3 Fluorescence by Triclosan

The application of 3 μM triclosan to thymocytes shifted the Fluo-3 histogram toward the direction of higher fluorescence intensity as compared to control treated cells (Fig. 1A), indicating that triclosan increased intracellular the Ca^{2+} concentration. The effect of 3 μM triclosan on Fluo-3 fluorescence reached a steady-state level within 1–3 min after drug application (Fig. 1B). Increasing the dose of triclosan to 10 μM further shifted the histogram toward higher fluorescence intensity (Fig. 1A), demonstrating that the effect of 10 μM triclosan on intracellular Ca^{2+} concentration was more profound than 3 μM and indicates a dose-response effect of triclosan. This dose-response relationship between triclosan treatment and the induction of Ca^{2+}, as measured by Fluo-3 fluorescence, is summarized in Fig. 1C. On the lower end of the dose response curve, a significant increase in the intensity of Fluo-3 fluorescence over control cells was observed, beginning at the 1 μM dose of triclosan. Although the thymocyte cell viability in the presence of 10 μM triclosan decreased in a time-dependent manner, it did not significantly change within 10–15 min after the application of 10 μM triclosan; the cell viability under control conditions was 94.7 ± 0.5% and remained at 93.7 ± 0.9% at 15 min after the application of 10 μM triclosan. Triclosan at the 3-μM dose or below did not significantly affect cell viability during the 3-hr incubation (data not shown). Therefore, the subsequent experiments, described below, were mainly performed with 3 μM triclosan.

Effect on Membrane Ca^{2+} Permeability

To determine if the increase in membrane permeability to Ca^{2+} can explain the triclosan-induced increase in Fluo-3 fluorescence intensity, the effect of 3 μM triclosan on Fluo-3 fluorescence was studied when the concentration of $CaCl_2$ in the cell suspension was increased from 0.67 mM to 6.7 mM. The cells were incubated with $CaCl_2$ at the respective concentrations for 10–15 min before the application of triclosan. Dose-dependence on external Ca^{2+} was observed because as the concentration of $CaCl_2$ increased, the Fluo-3 fluorescence by the same dose of triclosan also increased and became more profound (Fig. 2A).

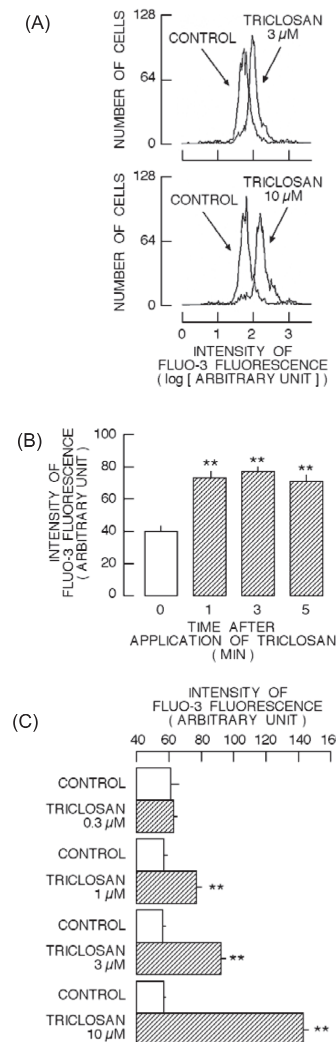

Fig. 1. Triclosan-induced Change of Fluo-3 Fluorescence in Thymocytes

(A) Histograms of Fluo-3 fluorescence. The effects of triclosan were examined 5 min after application. Each histogram was constructed with 2000 cells. (B) Time-dependent change in the intensity of Fluo-3 fluorescence. The time "0" indicates the control before the application of 3 μM triclosan. The symbol (**) shows significant change ($p < 0.01$) between the control group and the group of cells treated with triclosan. (C) Concentration-dependent changes in the Fluo-3 fluorescence intensity in response to triclosan. The effect was examined 5 min after the start of triclosan application. The column and bar indicate mean and standard deviation, respectively, of 3 experiments. The symbol (**) indicates a significant change ($p < 0.01$) between the control group and the group of cells treated with triclosan.

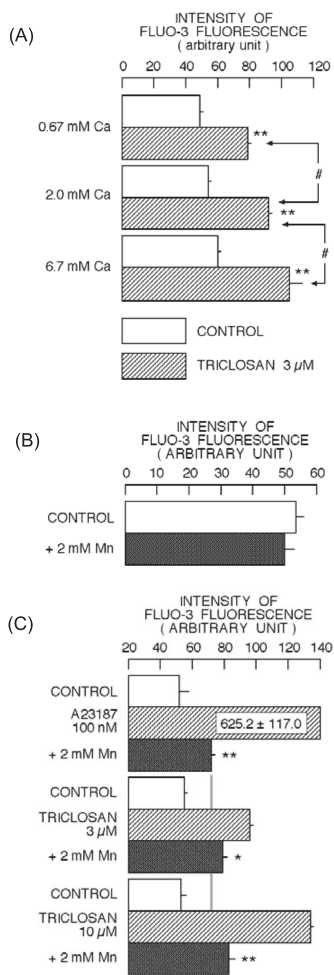

Fig. 2. Increase in Membrane Permeability to Ca^{2+} by 3 μM Triclosan

(A) Triclosan-induced augmentation of Fluo-3 fluorescence at different concentrations of external Ca^{2+}. The effects were examined 5 min after the start of triclosan drug application. The column and bar indicate mean and standard deviation, respectively, of 3 experiments. Symbols (∗∗) show significant change ($p < 0.01$) between the drug-treated groups and respective control groups. Symbol (#) indicates significant difference ($p < 0.05$) between paired groups. (B) Effect of $MnCl_2$ on Fluo-3 fluorescence of untreated control cells. The concentration of $CaCl_2$ used in this experiment was 2 mM. (C) Effects of $MnCl_2$ on Fluo-3 fluorescence of the cells treated with A23187 or triclosan. Notably, 100 nM A23187 increased the intensity of Fluo-3 fluorescence to 625.2 ± 117.0. The effect of $MnCl_2$ was examined 5 min after the start of its application. Before the application of $MnCl_2$, the cells were treated with triclosan or A23187 for 5 min. Symbols (∗, ∗∗) show significant change ($p < 0.05$, $p < 0.01$, respectively) between the drug-treated groups with and without $MnCl_2$. Dotted line indicates the intensity of Fluo-3 fluorescence of A23187-treated cells after $MnCl_2$-induced quenching.

To test if the increase in membrane permeability by triclosan is specific for Ca^{2+}, the effect of another divalent cation, Mn^{2+}, on the triclosan-induced increase in the Fluo-3 fluorescence intensity was examined by treatment with 2 mM $MnCl_2$ at 5 min after triclosan application. Permeability can be assessed because Mn^{2+} can replace the Ca^{2+} that is bound to Fluo-3, resulting in fluorescence quenching.[25] As shown in Fig. 2B, the application of 2 mM $MnCl_2$ to control thymocytes also treated with 2 mM $CaCl_2$ in the absence of triclosan did not significantly change the intensity of Fluo-3 fluorescence, indicating that divalent cation influx into the cell is well-regulated at baseline. A23187 was used in this study as a positive control reference agent because it nonspecifically increases membrane permeability of divalent metal cations.[26] As predicted, treatment with A23187 drastically increased the intensity of Fluo-3 fluorescence, while the further addition of 2 mM $MnCl_2$ significantly quenched the Fluo-3 fluorescence (Fig. 2C) because of the increased permeability. Similar to treatment with the permeabilization positive control, $MnCl_2$ at 2 mM also quenched the Fluo-3 fluorescence augmented by 3–10 μM triclosan (Fig. 2C). The results indicate that triclosan increased membrane permeability to not only Ca^{2+} but also other divalent cations like Mn^{2+}.

Intracellular Source of Increased Ca^{2+}

As described above, external Ca^{2+} participated in the triclosan-induced augmentation of Fluo-3 fluorescence. Therefore, the effect of triclosan on Fluo-3 fluorescence was examined under conditions free from external Ca^{2+} to determine if triclosan still had any effect. Triclosan at the 3 μM dose still increased the Fluo-3 fluorescence intensity under this external Ca^{2+}-free condition (Fig. 3A). A steady state of Fluo-3 fluorescence after triclosan treatment was achieved within 5 min after triclosan application (Fig. 3B). This increase by triclosan in the external Ca^{2+}-free condition was less than that under the normal Ca^{2+} concentration condition. The dose-response relationship between triclosan treatment and the augmentation of Fluo-3 fluorescence under the external Ca^{2+}-free condition is shown in Fig. 3C. The significant increase in the Fluo-3 fluorescence intensity by triclosan under the external Ca^{2+}-free condition was also observed at doses of 1 μM or more.

To see if Ca^{2+} released from intracellular stores is also involved in the triclosan-induced increase in Fluo-3 fluorescence, the effect of 3 μM triclosan was tested on the Fluo-3 fluorescence of cells treated with 100 nM A23187 under conditions free from external Ca^{2+}. Under the external Ca^{2+}-free condition, A23187 depletes Ca^{2+} in intracellular Ca^{2+} stores.[27,28] Although A23187 increased

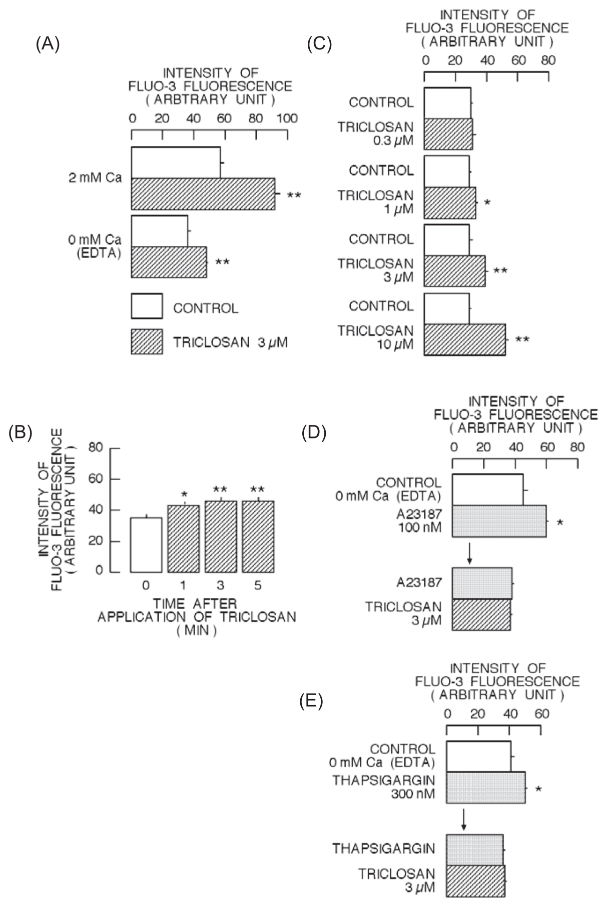

Fig. 3. Increase in Intracellular Ca^{2+} Release by Triclosan

(A) Effect of 3 μM triclosan on Fluo-3 fluorescence under the external Ca^{2+}-free condition. The effect was examined 5 min after the start of triclosan drug application. The column and bar indicate mean and standard deviation, respectively, of 3 experiments. The symbol (∗∗) shows significant change ($p < 0.01$) between the control group and the group of cells treated with triclosan. (B) Time-dependent change in the intensity of Fluo-3 fluorescence under the external Ca^{2+}-free condition. The time "0" indicates the control before the application of 3 μM triclosan. Symbols (∗, ∗∗) show significant change ($p < 0.05$, $p < 0.01$, respectively) between the control group and the group of cells treated with triclosan. (C) Concentration-dependent changes in the intensity of Fluo-3 fluorescence under the external Ca^{2+}-free condition. The effect was examined at 5 min after the start of drug application. Symbols (∗, ∗∗) show significant change ($p < 0.05$, $p < 0.01$, respectively) between the control group and the group of cells treated with triclosan. (D) Effect of triclosan on Fluo-3 fluorescence of the cells treated with A23187 under the external Ca^{2+}-free condition. The effect of triclosan was examined at 5 min after the start of triclosan drug application. Before the application of triclosan, the cells were treated with A23187 for 10 min. Symbol (∗) shows significant change ($p < 0.05$) between the control group and the group of cells treated with A23187 or triclosan. (E) Effect of triclosan on Fluo-3 fluorescence of the cells treated with thapsigargin under external Ca^{2+}-free condition. The effect of triclosan was examined 5 min after the start of triclosan drug application. Before the application of triclosan, the cells were treated with thapsigargin for 10 min. The symbol (∗) shows significant change ($p < 0.05$) between the control group and the group of cells treated with thapsigargin or triclosan.

the Fluo-3 fluorescence intensity in thymocytes, the fluorescence intensity returned to control levels or even lower within 10 min in the continued presence of A23187. Triclosan treatment of the 3 μM dose did not change the intensity of Fluo-3 fluorescence in the cells treated with A23187 (Fig. 3D). Similarly, in cells treated with 300 nM thapsigargin, an inhibitor for endoplasmic reticulum Ca^{2+}-ATPase, no change is observed in Fluo-3 fluorescence intensity (Fig. 3E). These results indicate that the increased Ca^{2+} levels by triclosan treatment under the external Ca^{2+}-free condition was derived from the endoplasmic reticulum stores.

DISCUSSION

Effect of Triclosan

Because of the following evidence, it is likely that triclosan at doses of 1 μM or more increases intracellular Ca^{2+} concentration by increasing membrane Ca^{2+} permeability and releasing Ca^{2+} from intracellular store(s) in rat thymocytes. The augmentation of Fluo-3 fluorescence by triclosan became more profound as the membrane gradient of Ca^{2+} increased by increasing external Ca^{2+} concentration (Fig. 2A), whereas the removal of external Ca^{2+} greatly attenuated the augmentation of Fluo-3 fluorescence by triclosan (Fig. 3A). Thus, it is likely that triclosan increases the external Ca^{2+} that passes through the cell membranes. The application of Mn^{2+} quenched the Ca^{2+}-dependent Fluo-3 fluorescence augmented by triclosan (Fig. 2C), indicating that Mn^{2+} passed across the membranes. Taken together, the data suggest that triclosan can increase membrane permeability to divalent metal cations. Triclosan increased the intensity of Fluo-3 fluorescence under conditions free from external Ca^{2+} (Fig. 3A). However, triclosan did not increase the Fluo-3 fluorescence intensity under the external Ca^{2+}-free condition when Ca^{2+} was depleted from intracellular Ca^{2+} stores by A23187 (Fig. 3D). Therefore, the data suggests that triclosan can release Ca^{2+} from intracellular stores.

In the study by Ahn *et al.*,[17] a micromolar triclosan dose was shown to enhance the binding of ryanodine to its receptor and augment the fluorescence of Fura-2, an indicator for intracellular Ca^{2+}, in mouse skeletal myotubes. The results suggested that triclosan induced release of Ca^{2+} from intracellular sarcoplasmic reticulum stores. As shown in Fig. 3, micromolar dose of triclosan induced the augmentation of Fluo-3 fluorescence in rat thymocytes, also suggesting the release of Ca^{2+} from in-

tracellular stores. Therefore, the intracellular Ca^{2+} release may be one of the common cytotoxic effects of triclosan. If triclosan depletes Ca^{2+} from endoplasmic reticulum, it would activate an intracellular-store-operated Ca^{2+} influx.[29–32] The introduction of $MnCl_2$ quenched the Fluo-3 fluorescence augmented by triclosan (Fig. 2C), indicating that Mn^{2+} was able to permeate across the membranes. This Mn^{2+} influx provides a qualitative evidence for the activation of store-operated Ca^{2+} entry.[31, 32] Therefore, triclosan may activate the intracellular-store-operated Ca^{2+} influx by depleting Ca^{2+} from the endoplasmic reticulum of rat thymocytes.

Implications

Since intracellular Ca^{2+} plays a physiological role as a second messenger,[18, 19] the increase in intracellular Ca^{2+} concentration by triclosan may disturb the carefully regulated intracellular Ca^{2+} homeostasis and lead to cellular malfunctions. The primary function of thymocytes is to generate T lymphocytes (T cells). The function of T cells is to recognize foreign antigens, and this is mediated by the T cell receptor. The thymocyte cells that fail to produce a functional receptor are eliminated by apoptosis. Apoptosis is the fate of most thymocytes.[33] It has become clear that the cellular Ca^{2+} overload or perturbation of the intracellular Ca^{2+} compartmentalization can cause apoptotic cell death.[34] Thus, triclosan may affect the process of apoptosis in thymocytes, resulting in a change in the competency of the immune system. Therefore, the main concern of the data presented here is whether the concentrations of triclosan detected in humans and wild animals reach a level that can increase intracellular Ca^{2+} concentrations. Since human plasma concentrations of triclosan[3] seem to be in the submicromolar ($< 0.131\ \mu M$) range, it is unlikely that this triclosan concentration could affect intracellular Ca^{2+} concentrations. However, the concentration of triclosan found in the maternal milk and bile of wild living fishes were 60–300 μg/kg lipid weight (0.207–1.036 μmol/kg lipid weight) and 0.24–0.9 mg/kg fresh weight (0.828–3.108 μmol/kg fresh weight), respectively, indicating that triclosan may be accumulating in certain lipophilic tissues.[11] The possibility that triclosan can increase intracellular Ca^{2+} concentration cannot be ruled out when triclosan accumulates in the tissues of humans and wild animals. At present, however, there is not sufficient evidence for adverse effects of triclosan on wild animals and humans. Therefore, further studies should be performed to determine whether adverse effects of triclosan exposure occur in nature.

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