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Antimicrobial Agent Triclosan Disrupts Mitochondrial Structure, Revealed by Super-resolution Microscopy, and Inhibits Mast Cell Signaling via Calcium Modulation

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Abstract

The antimicrobial agent triclosan (TCS) is used in products such as toothpaste and surgical soaps and is readily absorbed into oral mucosa and human skin. These and many other tissues contain mast cells, which are involved in numerous physiologies and diseases. Mast cells release chemical mediators through a process termed degranulation, which is inhibited by TCS. Investigation into the underlying mechanisms led to the finding that TCS is a mitochondrial uncoupler at non-cytotoxic, low-micromolar doses in several cell types and live zebrafish. Our aim was to determine the mechanisms underlying TCS disruption of mitochondrial function and of mast cell signaling. We combined super-resolution (fluorescence photoactivation localization) microscopy and multiple fluorescence-based assays to detail triclosan's effects in living mast cells, fibroblasts, and primary human keratinocytes. TCS disrupts mitochondrial nanostructure, causing mitochondria to undergo fission and to form a toroidal, "donut" shape. TCS increases reactive oxygen species production, decreases mitochondrial membrane potential, and disrupts ER and mitochondrial Ca^{2+} levels, processes that cause mitochondrial fission. TCS is 60x more potent than the banned uncoupler 2,4-dinitrophenol. TCS inhibits mast cell degranulation by decreasing mitochondrial membrane potential, disrupting microtubule polymerization, and inhibiting mitochondrial translocation, which reduces Ca^{2+} influx into the cell. Our findings provide mechanisms for both triclosan's inhibition of mast cell signaling and its universal disruption of mitochondria. These

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mechanisms provide partial explanations for triclosan's adverse effects on human reproduction, immunology, and development. This study is the first to utilize super-resolution microscopy in the field of toxicology.

Graphical Abstract

Keywords

triclosan; mast cell; mitochondria; super-resolution microscopy; RBL-2H3; NIH-3T3; keratinocyte; Ca^{2+} ; fluorescence photoactivation localization microscopy (FPALM); reactive oxygen species; membrane potential; degranulation; microtubules

Introduction

Triclosan (TCS) is a broad spectrum antimicrobial (Lyman and Furia 1968) recently banned from some soap products following the FDA's 2016 risk assessment (Kux 2016). However, TCS remains in many high-production consumer products (*e.g.*, toothpaste, hand sanitizer) and in hospital soaps (Cooney 2010).

TCS is readily absorbed through the skin (Moss et al. 2000), oral mucosa (Gilbert et al. 1987; Lin 2000), and gastrointestinal tract (Sandborgh-Englund et al. 2006). Consumer products contain high concentrations of TCS, up to ~10 mM, and 3–25% of applied TCS is rapidly absorbed into the oral mucosa or skin (Gilbert 1987; Lin 2000; Moss et al. 2000), where it remains, largely in the parent form, for at least 24 hours (Manevski et al. 2015; Moss et al. 2000). Approximately 0.4 to 64 nmol of TCS per milligram of human skin tissue protein is found in human skin following one hour exposure to a typical consumer product (Manevski et al. 2015; Moss et al. 2000; Weatherly and Gosse 2017). In the current study, exposure of cells to micromolar TCS levels results in approximately 0.8–2.8 nmol TCS/mg

cell protein (Weatherly and Gosse 2017), on the low end of levels in exposed human tissue and similar to the 2.5 nmol TCS/mg protein dosing which caused mitochondrial toxicity in isolated rat mitochondria (Newton et al. 2005). In addition to high TCS levels within human tissues, high TCS concentrations have been found in human body fluids: 1 μ M in blood following ingestion of ~1 tablespoon of TCS mouthwash (Sandborgh-Englund et al. 2006) and up to 13.1 μ M in urine (Calafat et al. 2008). Thus, in this manuscript, we utilize TCS doses equivalent to levels found in human tissues directly exposed to TCS-containing consumer products (Weatherly and Gosse 2017).

While TCS is used in hospitals and in consumer products due to its positive clinical effects as an antibacterial (Daoud et al. 2014) and anti-gingival (Rover and Leu-Wai-See 2014) agent and also potentially could be used as a topical treatment for atopic dermatitis (Tan et al. 2010), recent epidemiological reports have linked TCS to a variety of human health problems. TCS exhibits detrimental effects on human thyroid function and reproduction (Koeppel et al. 2013; Velez et al. 2015; Wang and Tian 2015; Wang et al. 2015). TCS is also associated with increased occurrence of allergies and asthma in humans (Spanier et al. 2014). In boys, prenatal TCS exposure is associated with decreased head circumference (Philippat et al. 2014). However, the molecular mechanisms underlying these human effects are not yet known.

Resulting from efforts to determine the molecular effects of TCS on mammalian cells, our lab previously discovered both that TCS inhibits the functions of mast cells (Palmer et al. 2012; Weatherly et al. 2013), which are ubiquitous immune cells, and that TCS is a mitochondrial toxicant (Shim et al. 2016; Weatherly et al. 2016). However, the underlying mechanisms and the ultrastructural changes underlying these effects remain unknown, and this current study aimed to investigate these processes.

Recently, we and others (Ajao et al. 2015; Raftery et al. 2017) have reported that, at low micromolar doses, TCS is a proton ionophore mitochondrial uncoupler (which inhibits ATP production and increases oxygen consumption rate) in multiple types of living cells (Weatherly et al. 2016), including primary human keratinocytes (a major cell type in the outer layer of skin), and in zebrafish (Shim et al. 2016). The doses that cause this mitochondrial toxicity are non-cytotoxic (Palmer et al. 2012; Weatherly et al. 2016) and also non-lethal to zebrafish embryos (Shim et al. 2016). TCS possesses an ionizable proton as does other small-molecule mitochondrial uncouplers such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), and 2,4-dinitrophenol (DNP).

Along with inhibition of ATP production and enhancement of respiration, mitochondrial uncouplers also can affect mitochondrial (Rottenberg and Wu 1998) and plasma (Mohr and Fewtrell 1987b) membrane potential, Ca^{2+} levels (Mohr and Fewtrell 1987b), mitochondrial translocation (Theoharides et al. 2013), and reactive oxygen species (ROS) production (Okuda et al. 1992; Przygodzki et al. 2005). Donut-shaped or toroid mitochondria are observed in multiple cell types after exposure to CCCP and FCCP (Ding et al. 2012; Giedt et al. 2012; Liu and Hajnoczky 2011). Uncouplers also can disrupt the mitochondrial fusion/fission balance, leading to fragmentation (Giedt et al. 2012; Griparic et al. 2007). However,

TCS effects on mitochondrial ultrastructure, translocation, and Ca^{2+} dynamics are not yet known.

Another cell type in which we documented TCS-induced mitochondrial uncoupling is mast cells (Weatherly et al. 2016): immune effector cells involved in neurological, immunological, and cancer-related functions (Metcalf et al. 1997; Silver and Curley 2013). We have previously shown that TCS inhibits a variety of functions of mast cells, both in rat (Palmer et al. 2012; Weatherly et al. 2013) and human models (Weatherly et al. 2016). Mast cells are found in nearly all human tissues, including the oral mucosa and skin (Walsh et al. 1995; Walsh 2003), making them prime targets for TCS exposure. TCS inhibits multiple mast cell functions, including degranulation (Palmer et al. 2012; Weatherly et al. 2016), which is the release of chemical mediators (*e.g.*, histamine, serotonin, β -hexosaminidase) from the cell. Degranulation is initiated when antigen (Ag) binds to and crosslinks IgE-bound Fc ϵ RI receptors, leading to phosphorylation of kinases including Lyn and PLC γ (Kinet 1999). Inositol 1,4,5-triphosphate (IP3) is generated by PLC γ and binds to its receptor on the endoplasmic reticulum (ER) membrane, instigating a flood of Ca^{2+} out of the ER (Berridge 1993). Depletion of ER Ca^{2+} stores causes the ER Ca^{2+} sensor STIM-1 to bind to the Orai1 subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel in the plasma membrane (Vig et al. 2006), resulting in an influx of Ca^{2+} across the plasma membrane (Hogan et al. 2010) (store-operated calcium entry), SOCE (Putney 1986). Influx of Ca^{2+} across the plasma membrane permits reuptake of Ca^{2+} into the ER through sarco/endoplasmic Ca^{2+} -ATPase (SERCA) pumps (Ma and Beaven 2011). In mast cells, mitochondria support degranulation by acting as Ca^{2+} buffers, taking up Ca^{2+} from both the ER and the cytosol (Furuno et al. 2015; Takekawa et al. 2012). Cytosolic Ca^{2+} , along with ROS production (Swindle et al. 2004), activates protein kinase C (PKC), a key event leading to degranulation (Ozawa et al. 1993). Granules are transported to the plasma membrane via microtubules (Guo et al. 1998), for degranulation (Smith et al. 2003). Mitochondria also rely on microtubules for transport (Iqbal and Hood 2014), and degranulation requires translocation of mitochondria from around the nucleus to exocytotic sites on the plasma membrane (Zhang et al. 2011). Together, all of these processes lead to degranulation. However, TCS effects on ER/mitochondrial/cytosolic Ca^{2+} levels, mitochondrial translocation, ROS, and microtubules are not yet known, and the mechanism(s) underlying TCS inhibition of degranulation are not yet known.

Numerous critical biological processes and structures occur at lengths that conventional microscopy techniques cannot resolve. In conventional fluorescence microscopy, large numbers of fluorescent molecules are visible at once, and diffraction blurs molecules closer than 200–250 nm apart, obscuring fine details. Fluorescence photoactivation localization microscopy (FPALM) is a super-resolution microscopy technique that circumvents the diffraction limit, allowing for ~10X improved spatial resolution (Hess et al. 2006). FPALM uses photoactivatable fluorescent probes, which are initially non-fluorescent (inactive). A low-intensity activation laser converts a small subset of inactive fluorophores into active ones, which are then imaged, localized to precisely determine their positions, and then photobleached, turning them off permanently. The remaining inactive fluorophores undergo the process of activation, imaging, localization, and photobleaching. This process is repeated until enough molecules have been localized to reveal a super-resolved image of the sample.

In the first usage of super-resolution microscopy in the field of toxicology, we have utilized FPALM's 10X higher resolution to demonstrate that TCS disrupts mitochondrial nanostructure in multiple cell types including mast cells and primary human keratinocytes. We also show that TCS disrupts multiple other cellular functions including ROS production, Ca^{2+} mobilization, membrane potential, mitochondrial translocation, and microtubule formation. Together, these results illustrate a mechanism by which triclosan inhibits mast cell degranulation and causes universal dysfunction of mitochondria.

Methods

Chemicals and reagents

TCS (99%; Sigma-Aldrich) and CCCP (VWR) were dissolved into aqueous buffers to deliver concentrations (5–20 μM TCS) previously proven to be mitotoxic and inhibitory of mast cells, while not cytotoxic, in Weatherly *et al.* 2013 and 2016 and in Palmer *et al.* 2012. DNP (Sigma-Aldrich) was freshly prepared daily, in cell culture water with 3.7 g/L sodium bicarbonate buffer (VWR), pH 7.4, followed by filtration (0.22 μm). DNP concentration was determined via UV-Vis spectrophotometry (extinction coefficient 12,100 M/L/cm at 400 nm (Peralta et al. 2010)). The components of Tyrodes buffer (which contains glucose) are those of Weatherly *et al.*, 2013.

Cell culture

Primary human keratinocytes (LifeLine Cell Technology), NIH-3T3 mouse fibroblasts, and RBL-2H3 ("RBL") mast cells were cultured as in Weatherly *et al.*, 2016. Phenol red-free media (Gibco) contains no glutamine, phenol red, HEPES, or sodium pyruvate; fetal bovine serum, gentamicin, and L-glutamine were added, and complete media was filtered (0.22 μm).

Our previous experiments showing that TCS is a mitochondrial uncoupler (Weatherly et al. 2016) were performed in the absence of glucose. Glucose was omitted from those experiments because, in the presence of glucose, cells (both immortalized (Crabtree 1928; Ibsen 1961) and primary (Wang et al. 1976)) rely on glycolysis to generate ATP and, therefore, bypass mitochondrial oxidative phosphorylation (the "Crabtree effect"). Thus, in order to probe effects of mitochondrial toxicants on oxidative phosphorylation (Marroquin et al. 2007), cells must be forced to undergo oxidative phosphorylation; this effect is accomplished experimentally by omitting glucose, which is replaced by glutamine plus galactose ("galactose media"). Under these conditions, virtually all cellular ATP is generated by oxidative phosphorylation fueled by catabolism of glutamine (an appropriate fuel source because it is abundant in plasma (Van Slyke et al. 1943)). The galactose (which is catabolized much more slowly than glutamine (Lowry and Passonneau 1969)) is included in the media in order to supply the pentose phosphate pathway, to support biosynthesis and detoxification (Reitzer et al. 1979).

Preparation of Dendra2-TOM20 construct to image the mitochondrial outer membrane

The Dendra2-TOM20 (translocase of the outer membrane) construct was used to image the outer mitochondrial membrane. Into a p-Dendra2-N plasmid (Clontech), a TOM20 localizer

was inserted into the multiple cloning site upstream from the Dendra2 open reading frame to create a TOM20-Dendra2-N plasmid. A forward primer containing a new HindIII site and a reverse primer containing a new AgeI site were developed and used to clone the TOM20 localizer, via PCR, from 15 day old C57BL/6 mouse hind limb DNA. The PCR product underwent a double-digest with HindIII and AgeI, followed by ligation of the purified PCR product into the Dendra2 plasmid and by sequencing for confirmation.

Fluorescence photoactivation localization microscopy (FPALM) imaging and processing

NIH-3T3 cells were transfected with the Dendra2-TOM20 construct via Lipofectamine 3000 (ThermoFisher Scientific) as per manufacturer's instructions, and RBLs and keratinocytes were transfected via Amaxa Nucleofection. The next day, cells were exposed to TCS in galactose media-bovine serum albumin (BSA) or Tyrodes-BSA (BT; recipe in Hutchinson et al. 2011).

Imaging began immediately after exposure and continued for up to one hour. Imaging was performed on an Olympus IX71 microscope, with a 561 nm laser (Sapphire, Coherent) used to activate and excite Dendra2 fluorescence. For details, see Supplement. Data from the EMCCD were analyzed using custom-built MATLAB (MathWorks Inc.) scripts for single color localization (Hess et al. 2006; Smith et al. 2010).

FPALM mitochondria analysis

To quantify the presence or absence of mitochondrial donuts, localized images were analyzed using a second custom-built MATLAB script. For detail, see Supplement.

Image analysis was also performed using NIH Fiji ImageJ software mitochondria calculator plug-in (Dagda et al. 2009). The images were extracted to binary (mitochondria-specific fluorescence was shown as black pixels), thresholded, and smoothed to resolve individual mitochondria. ImageJ's "analyze particles" option was used and uniformly thresholded for pixel size to decrease background. Measures of mitochondrial fission (mitochondrial major axis, perimeter, and elongation) were 1.) graphed over 1 hr with 20 minute binning (Fig. 3A–I) and 2.) cells exposed between 30 and 60 minutes were combined with normalization to control (Fig. 3J).

Reactive Oxygen Species Detection Assay

Reactive oxygen species (ROS) levels were assayed via instructions of the OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, Inc.). Briefly, 50,000 cells were plated per well of a black, clear bottom 96-well plate in phenol red-free media or keratinocyte media and incubated overnight at 37°C/5% CO₂. RBL cells were exposed to 0.1 µg/mL monoclonal anti-DNP mouse immunoglobulin E (IgE; Sigma-Aldrich) in media for 1 hr at 37°C/5% CO₂. Cells received 100 µL of either ROS dye (50 µM final concentration) or methanol vehicle (0.25% final concentration) in BT and were incubated for 30 mins at 37°C/5% CO₂. Following dye incubation, cells were washed with BT, then exposed to ± TCS or CCCP and ± DNP-BSA antigen (Ag) in BT. Using a microplate reader (Synergy 2, Biotek), fluorescence (485 ± 10 nm excitation, 528 ± 10 nm emission) was monitored immediately after exposure, at 37°C. With keratinocytes, the same assay was conducted, except without

IgE and Ag exposure. Data were analyzed first by averaging the replicates for each chemical concentration, and then the baseline value for 0 μ M treatment without Ag was subtracted from all data points. Next, these values were normalized to the last time point for 0 μ M TCS without Ag. The final values were plotted as a time series. Area under the curve (AUC) for each individual experiment was determined with Graphpad Prism, averaged, and normalized to 0 μ M TCS (no Ag).

Mitochondrial Ca^{2+} assay

In order to measure mitochondrial Ca^{2+} levels, RBL cells were transfected with pCMV CEPIA2mt (a gift from Masamitsu Iino; Addgene plasmid #58218) (Suzuki et al. 2014) via electroporation using Amaxa cell line Nucleofector kit T. After transfection, 100,000 cells/well were plated in phenolred-free media in 8-well plates (ibidi USA inc.) and incubated at 37°C/5% CO_2 overnight. The following day, cells were sensitized with 0.1 μ g/mL IgE for 1 hr in phenolred-free media (100 μ L/well). Next, cells were washed with BT and imaged in 200 μ L BT for baseline, followed by addition of 200 μ L of 2X Ag $\pm$ 2X TCS in BT. Images were taken with a confocal microscope, at 37°C via ibidi heating system (Olympus FV-1000, with an Olympus IX-81 inverted microscope, an oil-immersion 100X objective [NA 1.4], and a 30 milliwatt multi-argon laser [488 nm excitation, emission 505–605 nm bandpass filter]). In ImageJ, background was subtracted before F/F0 normalization of fluorescence values: baseline readings were normalized to the first baseline reading (time point = -6), and Ag $\pm$ TCS values were normalized to the first image immediately following Ag $\pm$ TCS addition (time point = 0). Values were plotted as percent increase over baseline. Number of oscillations was determined by the following guidelines: an oscillation must have at least a 15% increase on both sides of the low point, and the low and high point must be separated by at least 4 data points (2 mins).

Endoplasmic Reticulum Ca^{2+} assay

To measure ER Ca^{2+} levels, RBL cells were transfected with ER-GCaMP6-210 (Juan-Sanz et al. 2017), via electroporation using Amaxa cell line Nucleofector kit T. In phenol red-free media, 100,000 cells/well were plated into a black, clear bottom 96-well plate (Greiner Bio-One) after transfection and incubated at 37°C/5% CO_2 overnight. The following day, cells were sensitized with 0.1 μ g/mL IgE for 1 hr in phenol red-free media. Next, cells were washed with BT, and exposed to TCS $\pm$ Ag in Ca^{2+} -free BT. Fluorescence was monitored with 485 $\pm$ 10 nm excitation, 528 $\pm$ 10 nm emission. Background was subtracted out by averaging all values from the non-transfected cells and using that average for the y-axis baseline value.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential (MMP) was assayed with MitoTracker Red FM (ThermoFisher Scientific). In galactose media, 30,000 RBL cells/well were plated in a black, clear bottom 96-well plate and incubated 1 hr at 37°C/5% CO_2 . MitoTracker Red FM dye was diluted to 8 nM (0.16% DMSO vehicle) or 1.25 nM (0.02% DMSO) in both galactose media-BSA and a 30 μ M TCS galactose media-BSA solution; these two solutions were used to prepare TCS dilutions. CCCP solutions were also made in MitoTracker dye-containing galactose media-BSA. Cells were exposed to TCS or CCCP with 8 nM dye for 1 hr at 37°C.

In the microplate reader, fluorescence was monitored at 530 ± 25 nm and 645 ± 15 nm emission. For further analysis details, see Supplement.

ATP production and cytotoxicity assays

ATP production was measured in BT as described in Weatherly *et al.* 2016. For ATP experiments with DNP treatment, DNP was prepared in galactose media-BSA, and then cells were exposed to DNP for 1 hr at 37°C. DNP interfered with fluorescence of the cytotoxicity assay reagent provided by the Toxglo manufacturer, so the trypan blue assay was used to assess DNP cytotoxicity.

Cytosolic Ca^{2+} assay

To measure cytosolic Ca^{2+} levels in mast cells, RBL cells were transfected with pGP-CMV-GCaMP6f (a gift from Douglas Kim; Addgene #40755 (Chen et al. 2013)) via electroporation using Amaxa cell line Nucleofector kit T (Cohen et al. 2015). In phenol red-free media, 100,000 cells/well were plated in a black, clear bottom 96-well plate after transfection and incubated at 37°C/5% CO_2 overnight. The following day, cells were sensitized with 0.1 $\mu\text{g/mL}$ IgE for 1 hr in phenolred-free media. Next, cells were washed and exposed to TCS $\pm$ Ag in BT. Fluorescence was monitored with 485 ± 10 nm excitation, 528 ± 10 nm emission. A lactate dehydrogenase assay for cytotoxicity was conducted immediately after the 1 hr read, following the protocolin (Hutchinson et al. 2011). To determine AUC, the y-axis baseline was set to the average of the no-Ag wells in order to measure fluorescence due to Ag and TCS exposure only.

Confocal imaging of mitochondrial translocation

RBL cells (52,500/well) were plated in phenolred-free media in μ -Slide 8-well glass bottom plates (ibidi USA inc.) overnight at 37°C/5% CO_2 . The following day, cells were exposed to 0.1 $\mu\text{g/mL}$ IgE in galactose media for 1 hr. Cells were washed, then incubated with 8nM MitoTracker Red FM and 100 nM Hoechst 33342. Next, cells were washed, then exposed to galactose media-BSA $\pm$ Ag and ± 10 μM TCS. After exposure, an aliquot of supernatant was taken to assay for degranulation as described in Weatherly *et al.*, 2013. Next, cells were washed, then exposed to galactose media-BSA or BT for imaging. After imaging, another aliquot of supernatant was taken to assay for β -hexosaminidase release as a marker for cell death.

Mitochondrial translocation was imaged on the confocal microscope described in the “Mitochondrial Ca^{2+} assay” sub-section, with the following changes: Hoechst fluorescence (excitation 405 nm, emission 430–470 nm) and MitoTracker Red fluorescence (excitation 543 nm, emission 560–660 nm) were measured. Image acquisition speed was 10 $\mu\text{s/pixel}$. Cells were analyzed using a Fiji ImageJ software mitochondria plug-in (Dagda et al. 2009), to determine total fluorescence of Mitotracker Red around the nucleus. This number was divided by total Mitotracker Red FM fluorescence within that entire cell, subtracted from 1, and multiplied by 100%, to calculate the percentage of mitochondria translocated away from nucleus.

Confocal imaging of microtubules

RBL mast cells were transfected via Amaxa Nucleofection (as described in “FPALM imaging and processing”) with pIRESneo-EGFP- α Tubulin, a gift from Patricia Wadsworth (Addgene plasmid # 12298) (Rusan et al. 2001). To ibidi μ -Slide 8 well glass bottom plates were added 100,000 transfected RBL cells/well in phenol red-free media at 37°C/5% CO₂. The following day, cells were exposed to 0.1 μ g/mL IgE in phenol red-free media for 1 hr. Cells were washed, then exposed to $\pm$ Ag and $\pm$ 10 μ M TCS in BT for 15 minutes at 37°C/5% CO₂. Images were taken via Olympus FV-1000 laser scanning confocal, using the same settings as in “Mitochondrial Ca²⁺ assay.”

Tubulin Polymerization Assay

In vitro tubulin polymerization assays were performed according to the manufacturer’s instructions (Cytoskeleton, Inc).

Trypan Blue Exclusion Cytotoxicity Assay

Trypan blue assays were conducted as in Hutchinson et al. 2011. Cells were plated (2–3 million/well) in a 6-well plate and incubated for 1 hr at 37°C, 5% CO₂. Next, 1 mL of each 2X DNP solution (in galactose media-BSA) was added to the 1 mL galactose media in each well and exposed for 1 h at 37°C, 5% CO₂.

Statistical analyses

All analyses were done in Graphpad Prism. In experiments where triplicates were used (as in data from figures 4, 7, 8, and 12) data were scanned using a Q-test with a 95% confidence interval and, if present, outliers were excluded, leaving duplicates that were averaged. In case of samples with duplicates (as in data from figures 6, 9, and 11 d), raw data were always within 5% of each other. The biological replicates from at least three different experiments were averaged and used to determine the SEM across the three experiments. Significance levels for most experiments were assessed via one-way ANOVA with Tukey’s *post-hoc* test. Significance for toroid mitochondrial morphology was determined via a two sample t-test. Mitochondrial fission time series analysis was conducted via linear regression, and significance was determined compared to a slope of zero. Significance of mitochondrial Ca²⁺ “highest percentage increase” and “number of oscillations” was assessed via unpaired one-tailed t-test.

Results

Triclosan alters mitochondrial morphology in multiple cell types

FPALM nanoscopy (Hess et al. 2006), was utilized to provide precise images of mitochondrial morphology. A construct linking the photoactivatable fluorescent protein Dendra 2 to the outer mitochondrial membrane protein TOM20 was used. TCS, dissolved in galactose media, induces donut morphology in NIH-3T3 cells (Fig. 1). Analysis reveals a significant increase in the fraction of donut-shaped mitochondria over time with TCS exposure, compared to control (Fig. 1C).

These TCS/galactose experiments were repeated with RBL mast cells and primary human keratinocytes (Fig. 2C–F). While donut morphology is not widely seen, TCS causes fragmentation of mitochondria in these cell types (Fig. 2C–F). Mitochondrial perimeter, major axis, and elongation were quantified. Mitochondrial elongation is the inverse of circularity; a value of “1” corresponds to a round mitochondrion whereas a higher number corresponds to a more tubular structure (Dagda et al. 2009). TCS decreases all three of these parameters over time in RBLs and keratinocytes, compared to no changes in control, indicating mitochondrial fission (Fig. 3A–F). Data from cells exposed to TCS for 30 to 60 mins were combined, showing significant decreases in all mitochondrial shape parameters compared to control (Fig. 3J).

In order to determine whether a concomitant decrease in ATP is necessary to induce changes in mitochondrial morphology, these experiments were repeated with NIH-3T3 cells exposed to TCS in BT buffer containing glucose, which allows glycolysis to produce normal levels of ATP regardless of mitochondrial health (Weatherly et al. 2016). In the presence of glucose, TCS does not induce donut morphology in NIH-3T3 cell mitochondria (Fig. 2A–B), as it does in the presence of galactose (Fig. 1). However, in glucose-treated NIH-3T3 cells, TCS-exposed mitochondria appear shortened and rounded compared to control (Fig. 2A–B), with decreases in mitochondrial perimeter, major axis, and elongation (Fig. 3G–J). This result, that TCS induces mitochondrial fragmentation even in the presence of glucose, indicates that inhibition of ATP production is not the cause of triclosan’s induction of mitochondrial fragmentation.

We utilized clonogenic assays (Komissarova et al. 2005) under the experimental conditions used to generate the data in Figures 1–3. TCS causes neither apoptosis nor necrosis in NIH-3T3 cells or keratinocytes (Fig. S1) or RBL cells (Weatherly et al. 2016) even while it induces mitochondrial morphology deformations in these cells.

Triclosan increases reactive oxygen species production in multiple cell types

An increase in intracellular ROS is associated with mitochondrial fragmentation (Deheshi et al. 2015; Fan et al. 2010), leading us to hypothesize that TCS could induce an increase in ROS, resulting in mitochondrial fragmentation. Using RBL mast cells, we examined effects of TCS (for 1 hr) on ROS production both with and without Ag stimulation. Ag increases ROS production in RBL cells (Fig. 4A, C; significance indicated by *, Fig. S2A). TCS also increases ROS production in RBLs, in the absence of Ag stimulation (Fig. 4A, Fig. S2A), and in primary human keratinocytes (Fig. 4B and D). The fluorescence traces (Fig. 4D) from which summary AUC bar graphs (Fig. 4B) were calculated are shown for keratinocytes. TCS appears to mildly increase ROS production of Ag-stimulated cells, but this effect is not statistically significant (Fig. 4A, Fig. S2A).

Using RBL mast cells and the same conditions as in the TCS experiments, we also examined effects of the canonical uncoupler CCCP on ROS production (Fig. 4C, Fig. S2B). Interestingly, CCCP produces the opposite effect on ROS production, compared to TCS: CCCP does not affect ROS production of unstimulated mast cells, but CCCP dramatically *reduces* intracellular ROS of Ag-stimulated RBLs (Fig. 4C; significance indicated by #).

We confirmed that this increase in ROS production is caused neither by TCS interference with the fluorescent probe (Fig. S3A) nor by cytotoxicity in either RBL cells (Fig. S2C) or keratinocytes (Fig. S2D). We also confirmed that the ROS probe fluorescence is not photobleached during the measurements (Fig. S3B).

Triclosan affects mitochondrial Ca^{2+} in Ag-stimulated RBL-2H3 mast cells

Uncouplers affect mitochondrial Ca^{2+} levels (Rao et al. 2015; Samanta et al. 2014; Suzuki et al. 2014). We measured fluorescence of the construct CEPIA2mt (Suzuki et al. 2014) in RBL mast cells, with Ag stimulation, using confocal microscopy. The baseline fluorescence reading of the unstimulated cells fluctuated ~10–15% but remained essentially flat (Fig. 5A). After Ag stimulation, fluorescence readings increase 40% above baseline immediately after Ag addition, with the first peak at 2 min after Ag. Ag stimulation also causes mitochondrial Ca^{2+} levels to undergo slow oscillations, with about 8 mins between peaks of each oscillation (Fig. 5A, Ag). Upon Ag + 10 μM TCS exposure, initial peak fluorescence readings increase to 65%. Interestingly, beyond that first peak, the presence of TCS abolishes the oscillations that are seen with Ag alone (Fig. 5A, Ag + TCS).

Next, the CEPIA2mt fluorescence traces for individual cells (rather than the averaged data shown in Fig. 5A) were analyzed. The highest percentage increase in mitochondrial Ca^{2+} level (“highest peak”) of each individual cell trace was determined. TCS increases this “highest peak” value, compared to Ag alone (Fig. 5B). The highest percentage increase graph (Fig. 5B) has higher values compared to the peaks shown in the combined time-series graph due to variability in the timing of the peaks from cell to cell, as expected from previous mast cell studies (Millard et al. 1989). Also, TCS decreases the number of oscillations compared to Ag alone (Fig. 5B). Thus, TCS disrupts mitochondrial Ca^{2+} buffering capacity.

Triclosan alters ER Ca^{2+} levels in Ag-stimulated RBL-2H3 mast cells

To determine whether TCS affects ER Ca^{2+} , we transfected the fluorescent construct ER-GCaMP6–210 (Juan-Sanz et al. 2017) into RBL mast cells. In the absence of extracellular Ca^{2+} , Ag stimulation alone causes an immediate and robust decrease in ER Ca^{2+} (as shown in the representative fluorescence trace in Fig. 6A), as expected (Berridge 1993). The decrease reaches the lowest point at ~10 mins after Ag addition, followed by a slow, gradual increase in fluorescence, which indicates re-filling of ER stores. TCS causes a more rapid and sustained decrease in ER Ca^{2+} level compared to Ag alone (Fig. 6A). Triclosan’s enhanced depletion of ER Ca^{2+} continues throughout the 1 hr measurement period: TCS suppresses the gradual increase in ER-GCaMP6 fluorescence during the 10–60 min time points following Ag addition (Fig. 6A). AUC analysis quantifies this decrease in ER Ca^{2+} with Ag stimulation compared to no-Ag control and the further decrease in ER Ca^{2+} with TCS and Ag (Fig. 6B). Thus, TCS enhances Ag-stimulated depletion of ER Ca^{2+} stores.

To measure ER pH levels, we transfected the fluorescent construct ER-pHluorin into RBL mast cells (de Juan-Sanz et al. 2017). Neither Ag nor Ag + TCS affected ER-pHluorin fluorescence (data not shown), in stark contrast to the large effects caused by Ag and TCS on ER-GCaMP6–210 fluorescence (Fig. 6A, B). Thus, neither Ag nor Ag+TCS exposure

changes the pH of the ER, compared to control, supporting the conclusion that triclosan's effects on ER-GCaMP6-210 fluorescence are caused by changes in ER Ca^{2+} levels and not by a corruption of the fluorescent probe due to a change in ER pH.

Triclosan decreases mitochondrial membrane potential

To examine whether TCS affects MMP, we used MitoTracker Red FM dye, which accumulates in the mitochondria in an MMP-dependent fashion. A significant decrease in MMP is seen after a 1 hr exposure to 10 μM TCS (Fig. 7). CCCP (1 μM for 1 hr), a positive control for MMP inhibition (Rottenberg and Wu 1998) also decreases MMP (Fig. 7). Both 1 μM CCCP and 10 μM TCS decrease MMP by ~20%. This result is similar to our previous findings with ATP production and oxygen consumption rate, showing that the canonical mitochondrial poison CCCP is ~10X more potent than TCS as a mitochondrial uncoupler (Weatherly et al. 2016). Careful controls were conducted (see Methods) to overcome triclosan's and CCCP's interference with MitoTracker Red fluorescence (similar to CCCP's known interference with a plasma membrane potential probe (Mohr and Fewtrell 1987b)).

Triclosan modestly decreases ATP production in Ag-stimulated RBL-2H3 mast cells in the presence of glucose

In light of the discovery that TCS is a mitochondrial uncoupler in multiple cell types (Weatherly et al. 2016), including in RBL mast cells, the question arises of whether triclosan's inhibition of degranulation is solely caused by its mitochondrial inhibition. In order to determine what percentage of triclosan's inhibition of degranulation observed in glucose-containing BT (Weatherly et al. 2013) is due to ATP inhibition, we performed the ATP production experiment under the same conditions as in a degranulation assay using BT buffer (with glucose). Under these conditions, TCS (20 μM) causes an ~8% decrease in ATP, compared to an ~80% decrease in degranulation. Thus, only ~10% of the inhibition of degranulation is due to a decrease in ATP when glucose is present in BT buffer (Fig. 8).

Triclosan inhibits the cytosolic Ca^{2+} response to Ag stimulation in RBL-2H3 mast cells

Cytosolic Ca^{2+} mobilization is inhibited by other known mitochondrial uncouplers (Mohr and Fewtrell 1987b). We transfected the fluorescent construct GCaMP6 into RBL mast cells in order to measure cytosolic Ca^{2+} levels. We confirmed successful transfection (Fig. S4A), and we confirmed that TCS does not interfere with GCaMP fluorescence (Fig. S4B). GCaMP6 fluorescence was measured in the plate reader immediately after Ag stimulation $\pm$ TCS and graphed (Fig. 9A), from which AUC was calculated. Antigen stimulation increased GCaMP fluorescence in RBL cells by a factor similar to that found by other researchers (Furuno et al. 2015). Both 10 and 20 μM TCS decrease the Ca^{2+} response compared to control (Fig. 9B). TCS at 10 μM decreases AUC to 0.6 ± 0.1 (SEM) of the 0 μM control level, and 20 μM TCS decreases AUC to 0.51 ± 0.04 (SEM). There is a rapid initial rise in fluorescence immediately after Ag stimulation (Fig. 9A, 0 μM TCS), due to release from ER stores (compare first 10 min of "0 μM TCS + Ag" data from each of Figures 6A and 9A) (Holowka et al. 2012; Mohr and Fewtrell 1987a), followed by a decrease and plateau phase (Fig. 9A, 0 μM TCS) due to influx of Ca^{2+} across the plasma membrane (Mohr and Fewtrell 1987a). Triclosan greatly reduces this plateau (Fig. 9A, +TCS). Interestingly, the initial rise in Ca^{2+} is only slightly inhibited by TCS (Fig. 9A, +TCS). This observation aligns both with

our finding that TCS does not inhibit release of Ca^{2+} from the ER (Fig. 6) and with published data showing that CCCP exhibits similar effects on cytosolic Ca^{2+} (Mohr and Fewtrell 1987b). GCaMP transfection, TCS treatment, and/or Ag treatment cause no cytotoxicity (Fig. S5A, B), confirming that the decrease in GCaMP fluorescence in Fig. 9 is not due to cell death. These data indicate that TCS inhibits the influx of Ca^{2+} across the plasma membrane.

Triclosan inhibits mitochondrial translocation in Ag-stimulated RBL-2H3 mast cells

We used MitoTracker Red to label mitochondria and Hoechst dye to label nuclei of RBL mast cells in galactose media-BSA (Fig. 10). In resting RBL mast cells, the majority of mitochondria are located around the nuclei (Fig. 10Ai, Bi). In contrast, Ag stimulation causes mitochondria to translocate away from the nucleus (Fig. 10Aii, Bii). Addition of 10 μM TCS (with Ag stimulation) inhibits the translocation of the mitochondria, leaving the majority of the mitochondria localized around the nucleus (Fig. 10Aiii). Analysis shows that ~17% of the mitochondria are located away from the nucleus in control cells, compared to ~50% of the mitochondria translocated away from the nucleus in Ag-stimulated cells after 30 min Ag exposure (Fig. 10Aiv). TCS causes the percentage of mitochondria that translocate in Ag-stimulated cells to drop back to control levels, ~23% translocation (Fig. 10Aiv).

With glucose present, control and Ag-stimulated translocation is ~10% higher than in galactose experiments (Fig. S6 vs. Fig. 10Aiv). When glucose is present, TCS decreases Ag-stimulated mitochondrial translocation; however, this decrease is not as pronounced as in galactose media (Fig. S6 vs. Fig. 10Aiv). Mitochondrial translocation requires ATP, so a more pronounced decrease in translocation in conditions (+galactose, no glucose) that decrease ATP is expected. Thus, TCS inhibits mitochondrial translocation, whether or not glucose is present. These data indicate that a large decrease in ATP is not required for a significant decrease in mitochondrial translocation. Mitochondrial translocation is not required for *de novo* cytokine release (Zhang et al. 2012); indeed, TCS does not inhibit cytokine release (Fig. S7) even though it inhibits both mitochondrial translocation (Fig. 10) and degranulation.

We confirmed that robust degranulation responses occurred and were inhibited by TCS under the experimental conditions used in the mitochondrial translocation assays (Fig. S8A). Also, triclosan's inhibition of mitochondrial translocation is not due to cell death (Fig. S8B).

To compare the TCS effects to those of a canonical mitochondrial uncoupler, translocation experiments were performed with 2.4 μM CCCP (a dose equally effective at inhibiting ATP as 10 μM TCS (Weatherly et al. 2016)). CCCP inhibits Ag-stimulated mitochondrial translocation, but to a lesser extent than TCS (Fig. 10, compare Aiv to Biv).

Triclosan disrupts tubulin polymerization in Ag-stimulated RBL-2H3 mast cells

We hypothesized that TCS disrupts microtubule polymerization, thereby inhibiting degranulation and mitochondrial translocation. We utilized confocal microscopy and α -tubulin-EGFP to image microtubule formation in transfected RBL mast cells. In control resting cells, α -tubulin-EGFP is mainly located near the plasma membrane and in the

cytoplasm near the nucleus in a microtubule organization center (Joshi 1994) (Fig. 11A). Ag stimulation leads to drastic microtubule filamentation (Fig. 11B) in all 30 cells imaged (1 representative cell in Fig. 11B). TCS causes a drastic inhibition of microtubule formation in Ag-stimulated cells (Fig. 11C).

An *in vitro* (no cell) assay was conducted to determine whether TCS directly inhibits tubulin polymerization. No significant difference in tubulin polymerization is seen between control and 8.3 μM TCS (Fig. 11D); similar results were obtained with 1, 5, and 12 μM TCS (data not shown). A paclitaxel (inhibitor of microtubule disassembly) positive control (Fig. 11D) was included for comparison. These *in vitro* data show that TCS does not inhibit microtubule polymerization by directly binding to tubulin.

Triclosan is a more potent inhibitor of ATP production compared to the banned uncoupler 2,4-dinitrophenol

Assays of DNP effects on ATP production in RBL mast cells were performed, using the same experimental conditions used for TCS assays (glucose-free, galactose-containing media) (Weatherly et al. 2016). DNP disrupts ATP production in RBL cells with an EC_{50} value of 389–677 μM (95% CI) (Fig. 12). These concentrations are not cytotoxic (Fig. 12). Under these same conditions, we previously showed that TCS disrupts ATP production with an EC_{50} value of 7.5–9.7 μM (95% CI) (Weatherly et al. 2016). Thus, in RBL cells, TCS is ~60X more toxic to mitochondria than DNP. Analogous experiments were also conducted in primary human keratinocytes, leading to the conclusion that TCS is ~33X more mitotoxic than DNP in these cells (DNP EC_{50} 109–128 μM [95% CI]; TCS EC_{50} 3.0–4.1 μM [95% CI]).

Discussion

Triclosan is a proton ionophore mitochondrial uncoupler in numerous mammalian cell types (from different species and tissue types), including primary human skin cells, and in living zebrafish (Ajao et al. 2015; Shim et al. 2016; Weatherly et al. 2016), a well-accepted model for human toxicology (Planchart et al. 2016). By dissipating electrochemical gradients (Mohr and Fewtrell 1987b), proton ionophores interfere with both mitochondrial ATP production and a wide range of other cellular functions. In this study, we employed super-resolution microscopy among other techniques to detail cellular effects of TCS, at concentrations relevant to human exposures to consumer products, on mitochondria and on cellular signal transduction.

Using live-cell super-resolution microscopy for the first time in the field of toxicology, we show that micromolar doses of TCS dramatically and rapidly disrupt mitochondrial ultrastructure. TCS induces toroidal morphology and increases mitochondrial fission. Our results with TCS are supported by the fact that other known mitochondrial uncouplers also induce “donut” formation, by initiating mitochondrial swelling, detachment from microtubules, and auto-fusion (Liu and Hajnoczky 2011). FCCP and CCCP also increase fission (Giedt et al. 2012; Griparic et al. 2007). Although triclosan’s effects on mitochondrial morphology appear similar to changes caused during apoptosis (Sun et al. 2007), TCS does not cause apoptosis or necrosis under our experimental conditions (see

Supplement), and other uncouplers (FCCP and CCCP), likewise, do not cause apoptosis under conditions that cause fission/donuts (Griparic et al. 2007; Liu and Hajnoczky 2011). In fact, Liu and Hajnoczky also showed that mitochondria recover quickly after removal of FCCP (donut formation reversed), indicating that FCCP does not instigate an irreversible pathway leading to apoptosis under conditions in which it does distort mitochondrial shape (Liu and Hajnoczky 2011).

Mitochondrial shape is crucial for proper mitochondrial functioning, which directly impacts many diseases (Youle and van der Bliek 2012). For example, increases in occurrence of mitochondrial donuts in monkey presynaptic boutons are associated with cognitive decline (Hara et al. 2014). TCS has been detected in brain tissue of exposed fish (Escarrone et al. 2016) at 0.49 nmol TCS/mg protein, which lies within the 0.4–64 nmol TCS/mg protein levels found in human tissue following consumer product exposure (Weatherly and Gosse 2017). Certain genes linked to Parkinson's disease also increase the occurrence of mitochondrial donuts (Bhandari et al. 2014; Cui et al. 2010). In skeletal muscle, mitochondrial fission is associated with insulin resistance (Jheng et al. 2012). Mitochondrial fusion is essential for embryonic development (Chen et al. 2003), and a recent study found increased TCS levels in blood from mothers of infants with birth defects, compared to blood from mothers of healthy babies (Wei et al. 2017). Together, these findings suggest that triclosan's deformation of mitochondrial morphology could be a mechanism underlying disease.

In mast cells and keratinocytes, TCS increases ROS production, as found by other researchers investigating other species (Binelli et al. 2009; Riva et al. 2012; Tamura et al. 2012; Yueh et al. 2014). Because TCS increases ROS production in the presence of glucose, which ablates triclosan's inhibition of ATP production (in unstimulated cells) (Weatherly et al. 2016), triclosan's ROS production is not directly caused by mitochondrial uncoupling. A recent human epidemiology study shows a correlation between increased TCS in urine and an increase in a marker for oxidative stress (Lv et al. 2016). Triclosan's ability to increase ROS levels stands in contrast to other uncouplers, such as CCCP, which actually decrease ROS under some conditions (Przygodzki et al. 2005).

ROS production is required for mast cell degranulation (Swindle et al. 2004). Because TCS does not dampen mast cell ROS, manipulation of ROS is not the mechanism underlying triclosan's inhibition of degranulation (Weatherly et al. 2013). It should be noted that Ag stimulation causes a very strong stimulation of ROS levels, so an additional increase caused by TCS may be occurring, though difficult to measure. In fact, data in Fig. S2A do suggest a modest increase in Ag-stimulated ROS due to TCS.

We show, for the first time, that Ag stimulation induces slow oscillations in mast cell mitochondrial Ca^{2+} levels, as revealed by individual-cell analysis. TCS alters mast cell mitochondrial Ca^{2+} in an unexpected manner: TCS enhances the initial Ag-stimulated rise in mitochondrial Ca^{2+} but then abolishes subsequent oscillations. Thus, TCS may inhibit cytosolic Ca^{2+} levels and, hence, degranulation, by causing mitochondria to immediately sequester Ca^{2+} released by the ER following Ag stimulation. In contrast, FCCP and other uncouplers reduce mitochondrial Ca^{2+} (Rao et al. 2015; Samanta et al. 2014; Suzuki et al.

2014). This contrast between triclosan's and other uncouplers' effects on mitochondrial Ca^{2+} may be due to the fact that, unlike other uncouplers, TCS increases ROS, which is linked to increased mitochondrial Ca^{2+} levels (Jornot et al. 1999; Peng and Jou 2010). ROS increases mitochondrial Ca^{2+} levels by disrupting the $\text{Na}^+/\text{Ca}^{2+}$ ion exchanger, which is responsible for Ca^{2+} efflux from mitochondria (Jornot et al. 1999) (Palty et al. 2010). TCS-stimulated ROS may inhibit this exchanger and cause the initial enhancement of mitochondrial Ca^{2+} . The TCS-induced large initial mitochondrial Ca^{2+} peak may subsequently trigger the opening of the mitochondrial permeability transition pore (mPTP), leading to efflux of mitochondrial Ca^{2+} (Crompton 1999). The opening of the mPTP would also explain the lack of oscillations with TCS exposure. FCCP causes mPTP opening (Liu and Hajnoczky 2011). This FCCP-induced mPTP opening also may be a mechanism behind the mitochondrial donut formation (Liu and Hajnoczky 2011).

Mitochondria frequently are localized in close proximity to the ER (Mazel et al. 2009) in order to form mitochondrial/ER junctions (Pacher et al. 2000) that allow for Ca^{2+} transfer between mitochondria and the ER (Ma and Beaven 2011; Mazel et al. 2009). Recently, a role for the VAP family of ER membrane proteins in the initial rise in mitochondrial Ca^{2+} and for subsequent SOCE due to Ag stimulation was discovered (Holowka et al. 2016). Ag stimulation rapidly (within 2–4 min) lowers mast cell ER Ca^{2+} levels, as expected (Berridge 1993). TCS further drives down the Ag-stimulated decrease in ER Ca^{2+} , just as it enhances the Ag-stimulated increase in mitochondrial Ca^{2+} , indicating that TCS could be enhancing the Ca^{2+} exchange from the ER to the mitochondria. A possible explanation for this phenomenon is that TCS affects the ER's IP_3 receptor, leading to enhanced ER Ca^{2+} efflux. TCS increases production of ROS, which stimulates the IP_3 receptor (Suzuki and Ford 1992), enhancing Ca^{2+} release from the ER. Another possible mechanism behind triclosan's enhanced dampening of ER Ca^{2+} is that TCS-induced ROS production may inhibit the ER SERCA pump (Feissner et al. 2009), thereby inhibiting Ca^{2+} re-entry into the ER.

Triclosan's inhibition of MMP and enhancement of mitochondrial Ca^{2+} , together, illustrate a mechanism for TCS-induced mitochondrial fission. Lowered MMP leads to mitochondrial fragmentation (Giedt et al. 2012; Guillery et al. 2008). Elevation of mitochondrial Ca^{2+} induces mitochondrial fission in prostate cancer cells (Kaddour-Djebbar et al. 2010) and in human lung cells (Ahmad et al. 2013). Increases in cytosolic ROS correspond to mitochondrial fragmentation (Dehesi et al. 2015; Fan et al. 2010) and may also partially explain the TCS-induced mitochondrial fission.

In the presence of glucose, 20 μM TCS inhibits degranulation by 82% while inhibiting ATP by only 8%, indicating that mitochondrial uncoupling is not the only mechanism causing triclosan's inhibition of degranulation. Thus, TCS must target other components within the degranulation pathway. During mast cell degranulation, depletion of ER Ca^{2+} stores leads to CRAC channel activation and SOCE (Hogan et al. 2010). CRAC channel activation triggers mitochondrial translocation to the plasma membrane along microtubules (Quintana et al. 2006; Zhang et al. 2011). This mitochondrial translocation then, in turn, keeps the CRAC channels active, continuing the Ca^{2+} influx (Quintana et al. 2006). Our data indicate that TCS dampens Ag-stimulated degranulation, even in the presence of glucose, by decreasing cytosolic Ca^{2+} levels, via disruption of SOCE across the plasma membrane. Triclosan's

disruption of SOCE may be caused by its inhibition of mitochondrial translocation to the plasma membrane, through a decrease in microtubule polymerization. While TCS strongly inhibits microtubule polymerization in living cells, TCS does not directly interfere with tubulin polymerization *in vitro*. Inhibition of microtubule polymerization also impedes antigen-stimulated granule movement to the plasma membrane, thereby dampening degranulation (Smith et al. 2003). Because it is a proton ionophore, triclosan-induced disruption of the plasma membrane potential (PMP) is another potential contributor to the decrease in Ca^{2+} influx (Mohr and Fewtrell 1987b); however, we were unable to test PMP because TCS interferes with the fluorescence of plasma membrane potential probes. Triclosan's disruption of microtubules is similar to FCCP's microtubule disruption, caused by FCCP's inhibition of MMP (Maro et al. 1982); TCS could be acting in a similar manner. Any cell type that depends on calcium signaling, mitochondrial function, and cytoskeleton function (e.g., T cells, muscle cells) are subject to TCS effects (Feske 2007; Hill-Eubanks et al. 2011).

It is interesting to note that, under identical experimental conditions in RBL mast cells, TCS is a 60-fold more potent uncoupler, compared to the potent weight loss drug and uncoupler DNP (Tainter et al. 1934). Due to numerous adverse health effects, pharmacological usage of DNP was banned in the U.S. in 1938 (Harris and Corcoran 1995).

Several recent studies have found that TCS causes negative effects on human reproduction. One found decreased fecundity with increased TCS urine concentration (Velez et al. 2015). Other studies linked increasing TCS concentration in urine to miscarriage (Wang and Tian 2015; Wang et al. 2015). TCS reduces pregnancy weight gain and increases miscarriage in rats (Feng et al. 2016). Proper mitochondrial function is essential in reproduction (Ramalho-Santos et al. 2009), so triclosan's mitochondrial toxicity could partly explain these adverse reproduction effects. Human developmental defects associated with TCS exposure (Philippat et al. 2014; Wei et al. 2017) could be caused by triclosan's inhibition of mitochondrial fusion, which is essential for embryonic development (Chen et al. 2003; Chen and Chan 2010). Furthermore, a human study found an inverse relationship between urinary TCS concentration and body mass index (Li et al. 2015), which could be explained by triclosan's mitochondrial uncoupling. TCS-induced mitochondrial defects may also explain reduced energy and movement in exposed freshwater mussels (Goodchild et al. 2016). Triclosan's mitochondrial toxicity may underlie triclosan's adverse effects on reproduction; additional animal or human studies are required to prove a connection between triclosan's mitochondrial and human health effects.

In conclusion, using super-resolution microscopy, we have shown that TCS, a proton ionophore mitochondrial uncoupler (Weatherly et al. 2016), disrupts mitochondrial morphology in a rat mast cell line, a mouse fibroblast line, and primary human keratinocytes. We provide biochemical mechanisms underlying these effects, which occur at doses relevant to human exposure via consumer products. Also, TCS inhibits degranulation and other mast cell functions (Weatherly et al. 2013; Weatherly et al. 2016), and we have determined the mechanisms underlying this inhibition. This disruption of mitochondrial morphology is related to TCS-induced decrease in MMP and disruption of mitochondrial Ca^{2+} (Fig. 13). TCS-induced increases in ROS may disrupt the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ ion

exchanger, leading to increased mitochondrial Ca^{2+} levels, which, in turn, trigger the opening of the mPTP, leading to mitochondrial donut formation. Increased mitochondrial Ca^{2+} is also caused by enhanced ER Ca^{2+} depletion, which is caused by TCS. Mitochondrial uncoupling/dysfunction caused by TCS explains a portion but not most of triclosan's inhibition of mast cell degranulation. We additionally show that TCS inhibits mast cell degranulation via inhibition of Ag-stimulated influx of Ca^{2+} into the cytosol, enhancement of mitochondrial Ca^{2+} sequestration, and interference with mitochondrial Ca^{2+} oscillations (and possibly due to triclosan's interference with PMP). TCS also inhibits mast cell degranulation by decreasing MMP, which leads to disruption of microtubule polymerization, which causes inhibition of mitochondrial translocation, which ablates sustained activation of the CRAC channels (Fig. 13, adapted in part from Holowka et al. 2016). In summary, we show that TCS is a mitotoxicant that is much more potent than the banned drug DNP and also is a robust modulator of signal transduction in mammalian cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary of abbreviations

Ag	antigen
ATP	adenosine triphosphate
AUC	area under the curve
BSA	bovine serum albumin
BT	Tyrosine-bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CRAC	Ca^{2+} release-activated Ca^{2+}
DNP	2,4-dinitrophenol

ER	endoplasmic reticulum
FCCP	carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazine
FPALM	fluorescence photoactivation localization microscopy
IgE	immunoglobulin E
IP3	inositol 1,4,5-triphosphate
MMP	mitochondrial membrane potential
Mptp	mitochondrial permeability transition pore
PKC	protein kinase C
PMP	plasmamembrane potential
RBL	rat basophilic leukemia cells, clone 2H3
ROS	reactive oxygen species
SERCA	sarco/endoplasmic Ca ²⁺ -ATPase
SOCE	store-operated calcium entry
TCS	triclosan
TOM	translocase of the outer membrane
SEM	standard error of the mean

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Highlights

- Antimicrobial triclosan harms mast cells, fibroblasts, primary human keratinocytes
- Super-resolution microscopy reveals mitochondrial nanostructure deformation
- Triclosan causes mitochondrial fission via ROS, membrane potential, Ca^{2+} changes
- Triclosan inhibits mast cells via microtubule, mitochondrial, and Ca^{2+} changes

Fig. 1.

TCS exposure induces toroid morphology of NIH-3T3 mitochondria. Live cell super resolution FPALM microscopy of Dendra2-TOM20 labeled mitochondrial outer membrane. NIH-3T3 cells were exposed to 10 μ M TCS in galactose media-BSA for 1 hr. Shown are representative images (Control [A] and TCS [B]) taken from three independent days of imaging. Scale bars, 1 μ m. Analysis shows over time, 10 μ M TCS induces a toroid morphology in NIH-3T3 cell mitochondria compared to control (C). On the y-axis, ξ represents the fraction of regions that possessed a radial distribution function peak between 200 and 500 nm. Cells were binned in 21 minute intervals where exposure from 0–21 mins, 22–43 mins, and 44–60 mins were combined and plotted, as three data points per treatment. Values presented are means $\pm$ SEM of three independent experiments, which yielded a total of 9–13 cells tested per condition per time point. Two sample t-tests were performed, comparing control cells imaged within a specific time range to TCS-treated cells imaged

within the same specific time range, for each of the three binned time points. Statistically significant results are represented by * $p < 0.05$ and *** < 0.001 .

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Fig. 2. Super resolution FPALM microscopy of Dendra2-TOM20 labeled mitochondrial outer membrane in NIH-3T3 cells in glucose Tyrodes-BSA (A-B), RBL cells in galactose media-BSA (C-D), and primary human keratinocytes in galactose media-BSA (E-F). Live cell imaging was conducted the day following transfection of cells with the construct Dendra2-TOM20. Cells were exposed to $\pm 10 \mu\text{M}$ TCS (NIH-3T3 cells and keratinocytes) or $\pm 15 \mu\text{M}$ TCS (RBL cells). Shown are representative images of single cells taken from at least two independent days of imaging: control (A, C, E) and $10 \mu\text{M}$ (B, F) or $15 \mu\text{M}$ (D) TCS. Scale bars, $1 \mu\text{m}$.

Fig. 3.

TCS effects on mitochondrial perimeter, major axis, and elongation measurements in primary human keratinocytes (A-C, J), RBL cells (D-F, J), and NIH-3T3 cells (G-I, J). Responses were measured in RBL cells exposed to $\pm 15\mu\text{M}$ TCS in galactose media-BSA, keratinocytes exposed to $\pm 10\mu\text{M}$ TCS in galactose media-BSA, and NIH-3T3 cells exposed to $\pm 10\mu\text{M}$ TCS in glucose Tyrodes -BSA buffer. In (A-I), data were binned in 20 minute intervals from images which had been collected at exposure times from 0–20 mins, 21–40 mins, and 41–60 mins--plotted as three data points per treatment. In (J), data from cells

imaged between 30 and 60 mins after TCS exposure were combined for each cell type. Each cell type within each parameter was first normalized to the average of the corresponding control of that same cell type. These normalized values were then combined into a single control for each parameter measured. Values presented are means $\pm$ SEM; each binned interval includes data from 4 to 15 (A-I) or 10 to 22 (J) cells combined from at least 2 independent days of imaging. In (A-I), statistically significant results, as compared to a slope of zero, are represented by ** $p < 0.01$, as determined by linear regression. In (J), statistically significant results, as compared to control, are represented by ** $p < 0.01$ and *** $p < 0.001$, as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 4.

Intracellular reactive oxygen species production. ROS response was measured in RBL cells in a plate reader during stimulation with 0.01 $\mu\text{g/mL}$ Ag with 0, 5, or 20 μM TCS (A) or 0, 1, or 4 μM CCCP (C) and in keratinocytes with 0, 5, or 20 μM TCS exposure only (B, D), for 1 hr in Tyrodes-BSA. For (D), mean fluorescence intensity was determined by subtracting out the appropriate controls and normalizing to 0 μM TCS. The area under the curve was obtained from raw fluorescence values corresponding to ROS levels measured over a 1 hr period via Graphpad Prism software. AUC was plotted as a bar graph, normalized to control (no Ag, no TCS). Values presented are means $\pm$ SEM of at least three independent experiments; three replicates per treatment per experiment. Statistically significant results of $\pm$ Ag within a given toxicant dose are represented by * $p < 0.05$, *** $p < 0.001$ (A, C); of Ag + no CCCP vs. Ag + CCCP by ## $p < 0.01$, ### $p < 0.001$ (C); and of no-Ag control vs. no-Ag + TCS by †† $p < 0.01$, ††† $p < 0.001$ (A, B, D), as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 5.

TCS effects on mitochondrial Ca^{2+} levels in Ag-stimulated RBL mast cells. RBL cells were transfected with the CEPIA2mt construct to allow visualization of mitochondrial Ca^{2+} in individual cells, using confocal microscopy. Cells were exposed to $\pm 0.005 \mu\text{g/mL}$ Ag and $\pm 10 \mu\text{M}$ TCS in glucose Tyrodes -BSA buffer. Mitochondrial Ca^{2+} was analyzed as percentage increase in fluorescence after F/F_0 normalization (A). Baseline readings were taken for 6 minutes followed by simultaneous addition of Ag $\pm 10 \mu\text{M}$ TCS (at time point 0). Percent increase from lowest to highest fluorescence data point following Ag $\pm$ TCS addition was calculated for each individual cell, data were averaged, and significance of the TCS effect was determined via one-tailed t-test (B, black squares). Number of oscillations within a 15 minute period after Ag addition was determined as stated in the Methods section and

analyzed via one-tailed t-test (B, blue triangles). Values presented are means $\pm$ SEM of three independent days of imaging, with 14–22 cells analyzed per treatment. Statistically significant results compared to control are represented by ** $p < 0.01$, *** $p < 0.001$.

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Fig. 6.

TCS effects on ER Ca^{2+} levels in Ag-stimulated RBL mast cells in plate reader. RBL cells were transfected with the ER-GCaMP6–210 construct. Cells were exposed to $\pm 0.005 \mu\text{g/mL}$ Ag and $\pm 10 \mu\text{M}$ TCS in Ca^{2+} -free BT. Fluorescence was recorded for 1 hr immediately following Ag/TCS exposure: representative time-series (A). Average background fluorescence (from untransfected cells) was subtracted from each time point, and values presented are means $\pm$ SD of one representative experiment with three replicates per treatment (A). Area under this fluorescence curve (AUC) was determined using the raw fluorescence of the time-series graph, processed via background subtraction and normalization to 0 μM TCS/0 Ag (B). Values presented are means $\pm$ SEM of 3 independent experiments; two to three replicates for each experiment (B). Statistically significant results,

as compared to 0 μ M TCS + Ag, are represented by * $p < 0.05$, and, as compared to control (no stimulation), are represented by ### $p < 0.001$, determined by one-way ANOVA followed by Tukey's *post-hoc* test.

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Fig. 7.

Mitochondrial membrane potential of RBL cells. MMP response was measured in RBL cells after exposure to 0, 5, or 10 μ M TCS or $\pm$ 1 μ M CCCP for 1 hr in galactose media-BSA. Mean fluorescence intensity was determined by subtracting out appropriate controls and normalizing to 0 μ M Control. Values presented are means $\pm$ SEM of at least four independent experiments; three to four replicates per experiment. Statistically significant results, as compared to the appropriate control (0 μ M TCS or 0 μ M CCCP + DMSO vehicle), are represented by * $p < 0.05$, ** $p < 0.01$, as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 8.

ATP, degranulation, and cytotoxicity responses in RBL cells after TCS exposure in Tyrodes-BSA buffer. RBL cell responses were measured after 60 min total exposure to 0.0004 $\mu\text{g/mL}$ Ag $\pm$ TCS in Tyrodes -BSA. TCS concentrations ranged between 0 and 20 μM . The x-axis is a three-segment line to clarify data interpretation. Percentage of untreated control (y-axis) was determined by dividing the fluorescence or luminescence values of the sample by the average of the untreated control (ATP and cytotoxicity). These values were then normalized to control (0 μM TCS + Ag). Data presented are means $\pm$ SEM of at least three independent experiments; three replicates per experiment. The degranulation data is reproduced, with permission, from (Weatherly et al. 2013). Statistically significant results, as compared to control, are represented by * $p < 0.05$; *** $p < 0.001$, as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 9.

TCS effects on cytosolic Ca^{2+} levels in Ag-stimulated RBL mast cells. RBL cells were transfected with the GCaMP6 construct to allow measurement of cytosolic Ca^{2+} , using a plate reader. Cells were exposed to $\pm 0.005 \mu\text{g/mL}$ Ag and 0, 10, or 20 μM TCS in glucose Tyrodes-BSA buffer for 1 hr. (A) Representative graphs of GCaMP6 fluorescence, which was recorded for 1 hr immediately following Ag/TCS exposure. Average background fluorescence (from untransfected cells) was subtracted from each time point (A). Area under this fluorescence curve (AUC) was determined using the raw fluorescence of the time-series

graph, normalized to 0 μ M TCS (B). Values presented in (A) are means $\pm$ SD of one experiment; three replicates per treatment. Values presented in (B) are means $\pm$ SEM of at least 3 independent experiments; two to three replicates for each experiment. Statistically significant results, as compared to control, are represented by *** $p < 0.001$, determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 10.

TCS effects on mitochondrial translocation. Representative images of mitochondrial translocation in RBL mast cells with $\pm$ Ag and $\pm$ 10 μ M TCS (A) and $\pm$ Ag and $\pm$ 2.4 μ M CCCP (B) exposure in galactose media-BSA (images are pseudo-colored green for Mitotracker dye-labeled mitochondria and purple for Hoechst-stained nuclei). Cells were exposed to media only (Ai, Bi), media with 0.0004 μ g/mL Ag (Aii, Bii), or media with Ag and 10 μ M TCS or 2.4 μ M CCCP (Aiii, Biii) for 30 minutes at 37°C/5% CO₂. Scale bar, 10 μ m. Percentage of translocated mitochondria in control, Ag-stimulated, and Ag-stimulated + 10 μ M TCS or 2.4 μ M CCCP (Aiv, Biv) samples was quantified. Values presented (Aiv, Biv) are means $\pm$ SEM of three independent days of imaging. Figure Aiv combines data from control cells (total n=98), Ag-stimulated cells (n=73), and Ag-stimulated cells + TCS (n=81). Figure Biv combines data from control cells (n=84), Ag-stimulated cells (n=84), and Ag-stimulated cells + CCCP (n=90). Statistically significant results compared to Ag are represented by ***p < 0.001, statistically significant results compared to control are represented by ### p < 0.001, as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 11.

TCS effects on tubulin dynamics. Live cell confocal fluorescence microscopy of α -tubulin-EGFP transfected RBL cells (A-C). RBL cells were exposed to 0.005 μ g/mL Ag (B and C) and 10 μ M TCS (C) for 15 min with glucose Tyrodes-BSA, then imaged. One representative image from three independent days of imaging is shown. Scale bar, 10 μ m. Time-series graph of an *in vitro* tubulin polymerization assay, $\pm$ 8.3 μ M TCS or paclitaxel (D). Mean fluorescence intensity was determined by subtracting out the appropriate controls and normalizing to 0 μ M TCS. Values presented are means $\pm$ SEM of three independent experiments with two replicates per treatment per experiment. RFU values on the y-axis correspond to fluorescence values obtained from plate reader upon tubulin polymerization.

Fig. 12.

Cytotoxicity and ATP levels were measured in RBL cells after 60 min of DNP exposure in galactose media-BSA. DNP concentrations ranged from 0 to millimolar levels as indicated on the x-axis. The x-axis is a two-segment line to clarify data interpretation. Percentage of untreated control (y -axis) was determined by dividing the sample's signal by the average signal from the untreated control. These values were then normalized to control (0 μ M DNP). Data presented are means $\pm$ SEM of at least five independent experiments; three replicates per experiment. Statistically significant results, as compared to control, are represented by * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$, determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 13.

Schematic of TCS effects on mitochondria and on Ag-stimulated mast cell signaling.

Crosslinking of IgE-bound FcεRI receptors by multivalent Ag stimulates a phosphorylation cascade that leads to IP₃ generation. IP₃ then binds to its receptor on the ER membrane, causing efflux of Ca²⁺ from the ER, a process which is enhanced by TCS. While, in healthy cells, depletion of Ca²⁺ stores from the ER causes Stim1-Orai1 interaction and the activation of SOCE through plasma membrane CRAC channels, TCS instead leads to enhanced uptake of Ca²⁺ into mitochondria and inhibition of SOCE, leading to decreased cytosolic Ca²⁺ levels. Also, TCS inhibits microtubule polymerization and, thus, decreases mitochondrial translocation from the perinuclear region to the plasma membrane, further inhibiting SOCE. All these effects lead to inhibition of degranulation due to TCS exposure. TCS also decreases mitochondrial membrane potential (MMP), increases intracellular ROS, and modulates mitochondrial Ca²⁺ levels, all of which lead to fragmented mitochondria morphology. Red arrows indicate increases or decreases in various parameters, due to TCS exposure, and "X" marks indicate processes inhibited by TCS.