**Differential effects of triclosan on the activation of mouse and human peroxisome
proliferator-activated receptor alpha**

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Running Title: Triclosan Cytotoxicity and PPAR α Activation in Hepatic Cells

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Drug Administration.

Abstract

Triclosan is an anti-bacterial agent used in many personal care products, household items, medical devices, and clinical settings. Liver tumors occur in mice exposed to triclosan, a response attributed to peroxisome proliferator-activated receptor alpha (PPAR α) activation; however, the effects of triclosan on mouse and human PPAR α have not been fully evaluated. We compared the effects of triclosan on mouse and human PPAR α using PPAR α reporter assays and on downstream events of PPAR α activation using mouse hepatoma Hepa1c1c7 cells and human hepatoma HepG2 cells. PPAR α transcriptional activity was increased by triclosan in a mouse PPAR α reporter assay and decreased in a human PPAR α reporter assay. Concentrations of triclosan inhibiting 50% cell growth were similar in both human and mouse hepatoma cells. Western blotting analysis showed that triclosan increased acyl-coenzyme A oxidase (ACOX1), a PPAR α target, in Hepa1c1c7 cells but decreased the level in HepG2 cells. Treatment of Hepa1c1c7 cells with triclosan enhanced DNA synthesis and suppressed transforming growth factor beta-mediated apoptosis. This did not occur in HepG2 cells. These data demonstrate that triclosan had similar cytotoxicity in Hepa1c1c7 and HepG2 cells, but differential effects on the activation of PPAR α , the expression of ACOX1, and downstream events including DNA synthesis and apoptosis.

Keywords: Triclosan, PPAR α , DNA synthesis, apoptosis.

Introduction

Triclosan is a broad spectrum anti-bacterial agent widely used in a variety of personal care products, household items, medical devices, and clinical settings (Fang et al., 2010; Rodricks et al., 2010). Due to its extensive use and subsequent disposal into sewage systems, triclosan has been found in finished drinking water, surface water, waste water, and environmental sediments (Fang et al., 2010). Pharmacokinetic studies in humans and animals have shown that triclosan can be absorbed from both the gastrointestinal tract and skin (Rodricks et al., 2010). Therefore, there is potential that humans may be exposed to triclosan through dermal contact with personal care products containing triclosan or through the consumption of drinking water contaminated with triclosan. In fact, triclosan has been detected in human body fluids, including breast milk (Adolfsson-Erici et al., 2002; Dayan, 2007), plasma (Hovander et al., 2002), and urine (Calafat et al., 2008).

Long-term (> 18 months) oral exposure to triclosan leads to a significant increase in the incidence of hepatocellular adenomas and carcinomas in male and female mice (Rodricks et al., 2010). The activation of peroxisome proliferator-activated receptor alpha (PPAR α) has been proposed to be the mechanism for tumor induction. Indeed, several PPAR α activators, including cinnamyl anthranilate (NTP, 1980), trichloroethylene (NTP, 1988, 1990), and di(2-ethylhexyl)adipate (NTP, 1982), have been shown to induce liver tumors in mice. Triclosan has been shown to increase the number and size of peroxisomes and induce the expression of peroxisomal genes and fatty acid metabolism genes in mouse liver (Rodricks et al., 2010), suggesting that triclosan activated mouse PPAR α *in vivo*. However, there is still no direct evidence to support PPAR α activation by triclosan.

PPAR α is a ligand-activated transcription factor belonging to the nuclear receptor superfamily (Corton et al., 2014). The commonly described mechanism of PPAR α mediated transcription is that activation of PPAR α by endogenous or exogenous ligands results in the formation of a heterodimer with retinoid X-receptor (RXR) that binds to a sequence called the peroxisome proliferator response element (PPRE) within the promoter region of target genes (Berger and Moller, 2002; Chan and Wells, 2009; Rakhshandehroo et

al., 2010). Recently, it has become clear that the transcriptional regulation of target genes expression by nuclear receptor is a dynamic process (Hager et al., 2000; Hager et al., 2009; Hager et al., 2004; Stavreva et al., 2012). PPAR α plays a key role in the regulation of lipid metabolism and serves as a molecular target for hypolipidemic fibrate drugs (Lefebvre et al., 2006; van Raalte et al., 2004). The activation of PPAR α by peroxisome proliferators is a well-characterized mode of action of hepatocarcinogenesis in rodents (Corton, 2010; Corton et al., 2014; Peters et al., 2005).

In this study, we determined effect of triclosan on the activation of mouse PPAR α , using a PPAR α reporter assay. In addition, we evaluated the effects of triclosan on the protein levels of PPAR α and its target gene acyl-coenzyme A oxidase 1 (ACOX1), DNA synthesis, and transforming growth factor beta-mediated apoptosis in mouse hepatoma Hepa1c1c7 cells. We also investigated if the responses observed with mouse PPAR α and mouse hepatoma cells occurred with human PPAR α and human hepatoma cells.

Materials and Methods

Chemicals

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] was purchased from Alfa Aesar (Ward Hill, MA). GW7647 {2-[4-[2-[4-cyclohexylbutyl(cyclohexylcarbamoyl)amino]ethyl]phenyl]sulfanyl-2-methylpropanoic acid} was obtained from Calbiochem-Millipore (Billerica, MA). Transforming growth factor beta (TGF β), dimethyl sulfoxide (DMSO), propidium iodide, puromycin, thiazolyl blue tetrazolium bromide (MTT), etoposide, Williams' Medium E, and α -minimum essential medium were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin solutions and 2.5% trypsin were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). GW590735 {2-methyl-2-[4-[[[4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazole-5-carbonyl]amino]methyl]phenoxy]propanoic acid} was provided by Indigo Biosciences (State College, PA).

Antibodies

Goat polyclonal antibody to autophagy protein APG7 (APG7), mouse monoclonal antibodies to beclin 1 (BECN1), lysosome-associated membrane protein 2 (LAMP2), and β -actin, and rabbit polyclonal antibody to PPAR α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to acyl-coenzyme A oxidase 1 (ACOX1) was purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies to Golgi-associated PDZ and coiled-coil motif-containing protein PIST were obtained from ProSci, Inc. (Poway, CA). Rabbit polyclonal antibody to microtubule-associated protein 1 light chain 3 beta (LC3B) was purchased from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (clone B44) was from BD Biosciences (San Jose, CA).

PPAR α reporter assays

The transcriptional activity of mouse and human PPAR α was determined by measuring luciferase activity in PPAR α reporter assays (Indigo Biosciences, State College, PA) according to the manufacturer's instructions. The reporter cells, derived from Chinese hamster ovary cells, constitutively express mouse or human hybrid PPAR α , of which the DNA-binding domain (DBD) has been substituted with the DBD of GAL4, a transcription activator protein in yeast. These cells also incorporate a luciferase reporter gene driven by the GAL4 upstream activation sequence, which is the binding site for the DBD of GAL4. Thus, the effects of triclosan on the activation of PPAR α can be determined by measuring the luciferase activity.

Briefly, mouse or human PPAR α reporter cells were dispensed into the wells of a 96-well assay plate and immediately treated for 24 h with different concentrations of triclosan in the presence or absence of 20 nM GW590735 as a challenge activator. Control cells were treated with 0.1% (v/v) DMSO in compound screening medium. At the end of the treatment, the medium was discarded and the luciferase detection reagent was added. Luminescence was measured using a BioTek Synergy 2 Reader, and normalized to the cell viability, which was determined using an MTT assay. The half effective concentration

(EC₅₀) or the half inhibitory concentration (IC₅₀) on luciferase activity was analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

Cell culture and treatment

Mouse hepatoma Hepa1c1c7 cells and human hepatoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Hepa1c1c7 cells were cultured in α -minimum essential medium and HepG2 cells were grown in Williams' E medium. Both media were supplemented with 10% fetal bovine serum and antibiotics (100 U penicillin G/ml, 100 μ g streptomycin/ml, and 250 ng amphotericin B/ml). Both cell lines were maintained at 37°C in a humidified atmosphere with 5% CO₂. Triclosan (20 mM, stock solution) was dissolved in DMSO and stored at 4°C. Hepa1c1c7 cells were plated into the 24-well plates at cell densities of 1.6×10^4 , 8×10^3 , and 2×10^3 cells/cm² culture surface for 48, 72, and 120 h incubations, respectively. HepG2 cells were plated at a density of 6×10^4 , 4×10^4 , and 2×10^4 cells/cm² culture surface for 48, 72, and 120 h incubations, respectively. Cells were allowed to attach to the bottom of the wells for 24 h before being exposed to various concentrations of triclosan for 24, 48, or 96 h. Control cells were fed with complete culture medium containing 0.1 % (v/v) DMSO. For the 96 h cell treatment, fresh medium and triclosan were added every other day. Each of the incubations was performed three separate times, and all the measurements described below were conducted independently for each of the experiments.

The 293T cell line used for lentivirus packaging was purchased from Biosettia (San Diego, CA) and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, sodium pyruvate, non-essential amino acids, and antibiotics (Chen et al., 2014).

The half inhibitory concentration of triclosan on cell growth

The total number of viable cells was determined using an MTT assay as described previously (Wu et al., in press). Both cell lines were exposed to 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 μ M triclosan for 24, 48, or 96 h. At the end of the treatment, 100 μ l of MTT solution (5 mg/ml) was added to each well. After incubation at 37°C for 4

h, the medium was removed and 250 µl DMSO was added to each well to dissolve the formazan crystals. The absorbance of formazan was determined with a BioTek Synergy 2 reader (BioTek Instruments, Inc., Winooski, VT) at 540 nm, with 690 nm as the reference. The half inhibitory concentration of triclosan on cell growth was obtained from the cell growth curves, using GraphPad Prism 6.0.

Analysis of cell death

The number of necrotic cells was assessed using a lactate dehydrogenase (LDH) assay (Sigma) according to the manufacturer's instructions. The data from the LDH release assay were normalized by the total number of viable cells as assessed by an MTT assay (Fang and Beland, 2009). Markers of autophagic cell death, including BECN1, LAMP2, PIST, APG7, and LC3B, were analyzed using western blot as described below.

Apoptotic cell death was determined by caspase 3/7 activity, which was measured using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, at the end of the treatment, 100 µl of Caspase-Glo 3/7 reagent was added to each well of a 96-well plate containing 100 µl of culture medium per well. After incubation at room temperature for 30 min, luminescence was measured with a BioTek Synergy 2 Reader. An MTT assay was performed in a parallel 96-well plate. The luminescence was normalized to the MTT assay data, and further normalized to the control.

Western blot

Hepal1c7 cells and HepG2 cells were lysed with RIPA lysis buffer (Thermo Scientific, Rockford, IL) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The protein concentrations in the cell lysates were determined with a BCA Protein Assay kit (Thermo Scientific). Forty micrograms of cell lysate proteins was separated by SDS polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk and incubated with primary antibody against BECN1 (1:1,000), LAMP2 (1:2,000), PIST (1:1,000), APG7 (1:1,000), LC3B (1:2,000), PPAR α (1:1,000), ACOX1 (1:2,000), or β -actin (1:1,000) followed by a

secondary antibody conjugated to horseradish peroxidase. The blots were then detected by chemiluminescence using Immobilon Western Horseradish Peroxidase Substrate (Millipore Corporation, Billerica, MA). The intensity of each band was quantified by densitometry (UVP BioSpectrum AC Imaging System using VisionWorks LSD Image Acquisition & Analysis Software, Upland, CA), and the relative protein levels were calculated using β -actin as an internal reference.

BrdU incorporation assay

BrdU incorporation was determined by flow cytometric analysis. At the end of the treatment, the Hepa1c1c7 cells or HepG2 cells were pulse-labeled with BrdU by incubating the cells with complete medium containing 10 μ M BrdU for 1 h. The cells were then trypsinized, fixed in 70% ethanol, and stained with FITC-conjugated anti-BrdU monoclonal antibody and propidium iodide as previously described (Fang and Beland, 2009). The stained cells were analyzed with CellQuest software on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences).

Generation of Hepa1c1c7 cells with stable overexpression of mouse PPAR α

The coding sequence of mouse PPAR α was amplified from the plasmid pSG5 PPAR α (Addgene, Cambridge, MA, Plasmid 22751) (Tontonoz et al., 1994) and cloned into the cDNA expression lentiviral vector pLV-EF1a-MCS-IRES-Puro (Biosettia). 293T cells were co-transfected with the lentiviral packaging mix and the generated mouse PPAR α expression vector or the empty vector to produce lentivirus particles. Hepa1c1c7 cells were then infected with the lentivirus particles carrying mouse PPAR α expression vector or the empty vector, followed by the selection of Hepa1c1c7 cells with stable expression of mouse PPAR α (Hepa1c1c7/PPAR α cells) or the empty vector (Hepa1c1c7/vector cells) using puromycin (4 μ g/ml).

RNA interference of human PPAR α in HepG2 cells

Validated human PPAR α siRNA (siRNA ID 5348) and negative control siRNA were purchased from Life Technologies (Carlsbad, CA). Transfection of siRNA into HepG2 cells

was conducted by using Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's instructions. The knock-down efficiency of the PPAR α siRNA at the mRNA and protein level was determined using real-time PCR and western blot, respectively, 96 h after siRNA transfection.

RNA isolation and real-time PCR

At 96 h after siRNA transfection, total RNA was isolated from HepG2 cells using a RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA using a SuperScript III first-strand synthesis system (Life Technologies) following the manufacturer's instructions. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad laboratories) on a Bio-Rad CFX96 real-time PCR system. Pre-designed primers against human PPAR α and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* were obtained from Qiagen. All samples were run in triplicate. Data were normalized for the endogenous control *GAPDH* and analyzed using the $\Delta\Delta C_t$ method.

Comet assay

A comet assay, with or without post-incubation with bacterial formamidopyrimidine DNA glycosylase (Fpg) protein, was performed using a Fpg FLARE™ Assay kit or a CometAssay kit (Trevigen, Gaithersburg, MD), as described previously (Wu et al., in press; Wu et al., 2012). Briefly, following triclosan treatment for 48 or 96 h, or H₂O₂ treatment for 30 min as a positive control, floating and adherent Hepa1c1c7 cells and HepG2 cells were collected and washed three times in PBS. From the cell suspensions (1×10^5 cells/ml), a 10 μ l sample was removed and mixed with 100 μ l of low-melting-point agarose (37°C) to prepare a final cell-agarose suspension. From this suspension, 75 μ l was spread onto a frosted glass microscope slide pre-coated with a layer of normal-melting-point agarose. The slides were allowed to gel at 4°C for 45 min, and then immersed in pre-chilled lysis solution overnight at 4°C. After lysis, sample areas were treated with Fpg protein or left untreated at 37°C for 30 min. Slides with or without Fpg post-treatment were placed in alkali solution (pH > 13) at room temperature for 20 min to allow DNA unwinding and DNA strand breakage at alkali-labile sites, before electrophoresis in cold room for 25 min at 20 V and 300 mA

(Bio-Rad, Hercules, CA). After electrophoresis, the slides were immersed in ethanol, air-dried, and stained with SYBR Green. The fluorescence-labeled DNA was visualized (magnification, 200×) using a Nikon Eclipse 50i fluorescence microscope (Nikon Instruments Inc., Lewisville, TX) and the images were captured with image analysis software (Comet Assay IV, version 4.2, Perceptive Instruments, Suffolk, UK). At least 100 cells per slide were evaluated and the olive tail moment (tail length × percentage of DNA in tail) was used as the DNA damage parameter.

Statistical analyses

Data are represented as mean ± standard deviation of three independent experiments and analyzed by linear regression analysis and/or one-way analysis of variance followed by pairwise-comparisons using Dunnett's test. The difference was considered statistically significant when the *p* value was less than 0.05.

Results

Effects of triclosan on the activation of mouse and human PPAR α

The effects of triclosan on the activation of human and mouse PPAR α were initially examined using reporter assays. Reporter cells, derived from Chinese hamster ovary cells, exposed to triclosan (0.1 – 10 μ M) for 24 h did not display any cytotoxicity, while a decrease in cell viability was observed in cells treated with 20 μ M triclosan for 24 h, with the value being 64% of the control. As shown in Fig. 1A, triclosan exhibited potent mouse PPAR α activator activity after 24 h of exposure. The EC₅₀ value of triclosan on mouse PPAR α activation was 9.9 μ M (Fig. 1A). In contrast, triclosan not only decreased the basal luciferase activity, with an IC₅₀ of 14.3 μ M on human PPAR α (Fig. 1B), but also markedly suppressed the luciferase activity induced by the selective potent PPAR α activator GW590735 (20 nM), with an IC₅₀ of 13.9 μ M (Fig. 1C). These data indicate that triclosan acted as an activator of mouse PPAR α , but it did not have a similar effect on human PPAR α .

The half inhibitory concentrations of triclosan in Hepa1c1c7 cells and HepG2 cells

The differential effects of triclosan on the activation of mouse and human PPAR α was

investigated further by using mouse hepatoma Hepa1c1c7 cells and human hepatoma HepG2 cells. Initially, the cytotoxicity of triclosan in Hepa1c1c7 cells and HepG2 cells was determined. As shown in Fig. 2A, triclosan inhibited the cell growth of the Hepa1c1c7 cells and HepG2 cells in a concentration- and time-dependent manner. With Hepa1c1c7 cells, the half inhibitory concentrations were 44.6, 33.1, and 24.7 μ M after a 24, 48, and 96 h exposure, respectively (Fig. 2B). Although the half inhibitory concentrations in HepG2 cells following 24 and 48 h exposure were significantly different from that in Hepa1c1c7 cells, the half inhibitory concentrations obtained in both cell lines were comparable (Fig. 2B). These data clearly indicate that there was no substantial difference between Hepa1c1c7 cells and HepG2 cells in the inhibition of cell growth by triclosan.

Effects of triclosan on cell death

Based on the IC₅₀ values for cell growth, the concentrations of triclosan used in subsequent experiments were 2.5, 5, 10, and 20 μ M. The cytotoxicity of triclosan at these concentrations was first determined in both cell lines. Exposing Hepa1c1c7 cells or HepG2 cells to 2.5, 5, or 10 μ M triclosan for up to 96 h did not have any effects on the cell viability. However, a significant decrease in cell viability was observed in both cell lines treated with 20 μ M triclosan, with the cell viability being 90% in Hepa1c1c7 cells and 86% in HepG2 cells after a 48 h of exposure, and 65 % in Hepa1c1c7 cells and 28% in HepG2 cells for 96 h treatment.

The decrease in cell viability could be due to an increase in necrotic cell death, the induction of apoptosis, and/or an increase in autophagy. Each of these pathways was examined. As shown in Fig. 3A, a significant increase in LDH release was observed in Hepa1c1c7 cells after 96 h of exposure to 20 μ M triclosan and in HepG2 cells upon $\geq$ 48 h exposure to the same concentration of triclosan. Using caspase 3/7 activity as a measure of apoptosis, triclosan treatment for up to 96 h did not induce apoptotic cell death in either cell line, while 100 μ M etoposide, which was served as a positive control, significantly induced apoptosis in both cell lines (Fig. 3B).

Autophagy, a caspase-independent mechanism of programmed cell death, is a catabolic process characterized by self-ingestion of cellular components and proteins through autophagosomic-lysosomal machinery (He and Klionsky, 2009). To determine whether or not triclosan induced autophagy in Hepa1c1c7 cells or HepG2 cells, protein levels of several autophagy markers, including BECN1, LAMP2, PIST, APG7, and LC3B, were examined using western blot. Although the basal expression levels of these autophagy markers differed between the Hepa1c1c7 cells and HepG2 cells, no significant changes in the protein levels were observed by triclosan treatment in either cell line (Fig. 3C and 3D). These data indicate that triclosan treatment at concentrations $\leq 20 \mu\text{M}$ and 24 h of exposure was not cytotoxic to Hepa1c1c7 cells or HepG2 cells, and that the toxicity observed at $20 \mu\text{M}$ triclosan with a prolonged exposure ($\geq 48 \text{ h}$) was due to necrosis and not apoptosis or autophagy.

Effects of triclosan on the protein levels of PPAR α and ACOX1 in Hepa1c1c7 cells and HepG2 cells

To confirm the differential effects of triclosan on mouse and human PPAR α activation, the protein levels of PPAR α and ACOX1, a well-characterized PPAR α target, were measured in Hepa1c1c7 cells and HepG2 cells. Exposing the cells to triclosan for 24 h did not affect the protein levels of PPAR α in either cell line (Fig. 4). Triclosan treatment did produce a concentration-dependent increase ($p < 0.05$) in the protein levels of ACOX1 in Hepa1c1c7 cells, with the increase being significant at all concentrations (Fig. 4, left panel). In contrast, triclosan treatment resulted in a concentration-dependent decrease ($p < 0.05$) in the protein levels of ACOX1 in HepG2 cells with the decrease becoming significant at $20 \mu\text{M}$ triclosan (Fig. 4, right panel). These data indicate that triclosan causes a differential response in protein levels of ACOX1 in Hepa1c1c7 cells and HepG2 cells.

Effects of triclosan on DNA synthesis in Hepa1c1c7 cells and HepG2 cells

It has been suggested that both the induction of DNA synthesis and the suppression of apoptosis resulting from PPAR α activation contribute to liver tumorigenesis in rodents treated with PPAR α activators (Corton et al., 2014; Rodricks et al., 2010). Using a BrdU

incorporation assay, the effects of triclosan on DNA synthesis in Hepa1c1c7 cells and HepG2 cells were determined. In Hepa1c1c7 cells, triclosan increased the relative levels of BrdU positive cells in a concentration-dependent manner ($p < 0.05$) after 96 h of exposure, with an EC_{50} of 7.9 μ M; at shorter periods of exposure (< 96 h), triclosan had no effect on the relative levels of BrdU positive cells at any of the concentrations tested (Fig. 5A). In contrast to Hepa1c1c7 cells, exposing of HepG2 cells to 2.5, 5, 10, or 20 μ M triclosan for 96 h resulted in a concentration-dependent ($p < 0.05$) decrease in the relative levels of BrdU positive cells (Fig. 5B). Exposing cells to GW7647 (0.1, 1, or 10 μ M), a potent activator for both mouse and human PPAR α (Brown et al., 2001), led to an increase in the relative levels of BrdU positive cells in Hepa1c1c7 cells (Fig. 5C), but a decrease in HepG2 cells (Fig. 5D). These data indicate that triclosan treatment modulated DNA synthesis in opposite directions in Hepa1c1c7 cells and HepG2 cells.

Effects of PPAR α expression level on triclosan-mediated alteration in DNA synthesis in Hepa1c1c7 cells and HepG2 cells

To determine whether the effect of triclosan on DNA synthesis in Hepa1c1c7 cells and HepG2 cells is dependent on the expression level of PPAR α , we overexpressed mouse PPAR α in Hepa1c1c7 cells and knocked-down human PPAR α in HepG2 cells, and then determined the effect of triclosan on DNA synthesis in these cells in comparison with their corresponding parent cells. Hepa1c1c7/PPAR α cells exhibited 48-fold increase in PPAR α protein expression as compared to Hepa1c1c7 cells or Hepa1c1c7/vector cells (Fig. 6A). In HepG2 cells transfected with human PPAR α siRNA, the mRNA level of PPAR α decreased to 20% of the value in HepG2 cells transfected with negative control siRNA (Fig. 6B, right panel), and the protein level of PPAR α decreased to 60% of control (Fig. 6B, left panel), at 96 h after siRNA transfection. Although there was an extensive increase in PPAR α protein expression in Hepa1c1c7/PPAR α cells, following 96 h exposure, triclosan increased DNA synthesis in Hepa1c1c7/vector cells and Hepa1c1c7/PPAR α cells to similar extent (Fig. 6C, upper panel). Likewise, although the level of PPAR α mRNA was decreased in PPAR α siRNA transfected cells, the extent of triclosan-mediated decrease in DNA synthesis was also comparable between HepG2 cells transfected with PPAR α siRNA and HepG2 cells

transfected with negative control siRNA (Fig. 6C, lower panel).

Effects of triclosan on DNA damage

The increase in the relative levels of BrdU positive cells could be due to an increase in DNA replication or to S phase cell cycle arrest in response to the damaged DNA induced by triclosan. To evaluate whether or not triclosan induces DNA damage, alkaline comet assays were performed in which Hepa1c1c7 cells and HepG2 cells were incubated with various concentrations of triclosan for 48 and 96 h. As shown in Fig. 7, there was no significant increase in tail moment in either cell line in comet assays conducted with or without Fpg, a glycosylase from *E. coli* that has been widely used to assess oxidative DNA damage (Collins et al., 2008). For comparison, Hepa1c1c7 cells and HepG2 cells were also treated with 100 μ M H₂O₂ for 30 min. H₂O₂ clearly induced DNA damage in both the presence and the absence of Fpg (Fig. 7). These data suggest that triclosan did not induce DNA damage in Hepa1c1c7 cells or HepG2 cells, which in turn supports the concept that the increase in BrdU incorporation was due to an increase in DNA replication.

Effects of triclosan on TGF β -induced apoptosis in Hepa1c1c7 cells and HepG2 cells

The effects of triclosan on TGF β -induced apoptosis in Hepa1c1c7 cells and HepG2 cells were determined using a caspase 3/7 assay. It has been reported that TGF β induces apoptosis effectively in cells under reduced serum culture conditions (Goll et al., 2000; Gressner et al., 1997). After a 24-h attachment culture in 10% FBS medium, cells were cultured in 0.5% FBS medium containing triclosan (2.5 - 20 μ M) in the absence or presence of TGF β (10 ng/ml) for 24 and 48 h. After 24 h of exposure, triclosan did not cause any changes in caspase 3/7 activity in either cell line at any of the concentrations tested (data not shown). However, exposing of Hepa1c1c7 cells to triclosan for 48 h not only decreased the basal caspase 3/7 activity but also diminished the TGF β -induced caspase 3/7 activity in a concentration-dependent manner (Fig. 8). There was a significant increase in the caspase 3/7 activity in HepG2 cells exposed to 20 μ M triclosan for 48 h in the absence or presence of TGF β . Triclosan at concentrations $\leq$ 10 μ M in the absence or presence of TGF β had no

effect on the caspase 3/7 activity in HepG2 cells. These data indicate that triclosan treatment had differential effects on apoptosis between Hepa1c1c7 cells and HepG2 cells.

Discussion

In this study we demonstrated that triclosan exhibits differential effects on the activation of mouse and human PPAR α , which was supported by the following facts summarized in Table 1. Data from PPAR α reporter assay indicated that triclosan activated mouse PPAR α , but not human PPAR α . Furthermore, triclosan treatment increased PPAR α target ACOX1 expression, enhanced DNA synthesis, and suppressed TGF β -induced apoptosis in mouse hepatoma Hepa1c1c7 cells; however, the opposite effects were observed in human hepatoma HepG2 cells.

The activation of PPAR α is a well-characterized, non-genotoxic mode of action in rodent hepatocarcinogenesis. Species-dependent responsiveness to PPAR α activators has been reported previously. For instance, the well-characterized PPAR α activator WY-14,643 shows preferential activation for mouse PPAR α over human PPAR α (Keller et al., 1997), whereas 5,8,11,14-eicosatetraenoic acid is more potent in activating human PPAR α than mouse PPAR α (Keller et al., 1997). PPAR α has a domain structure consisting of an N-terminal activating function-1 domain, a central DNA-binding domain, which allows the physical interaction between PPAR α /RXR heterodimer and specific PPRE sequences, and a C-terminal ligand-binding domain, which is crucial for ligand binding and dimerization with RXR (Cronet et al., 2001; Hi et al., 1999). The amino acid sequence of human and mouse PPAR α shows 100% identity in the DNA-binding domain and 94% identity in the ligand-binding domain (Mukherjee et al., 1994). In spite of the overall high homology between mouse and human PPAR α , several amino acid residues in the ligand-binding domain have been demonstrated to be responsible for the preferential responsiveness to WY-14,643 or 5,8,11,14-eicosatetraenoic acid (Keller et al., 1997). Thus, the differential effects of triclosan on the activation of mouse and human PPAR α are probably due to different binding behaviors between triclosan and the ligand-binding domain of mouse and human PPAR α . Further mechanistic studies to understand the differences in binding behaviors between

triclosan and PPAR α using *in silico* molecular docking analysis and to identify the critical amino acid residues that mediate the binding of triclosan using site-directed mutagenesis are clearly warranted. It also should be noted that the reporter assays may overestimate the actual activity because in these assays chromatin remodeling or heterodimerization with RXR is not required for the binding of PPAR α to PPRE sequences.

In response to PPAR α activation, protein levels of ACOX1, which plays a key role in fatty acid metabolism and fat deposition (Rakhshandehroo et al., 2010), were induced in Hepa1c1c7 cells but decreased in HepG2 cells (Fig. 4). This was fully consistent with the results obtained in PPAR α reporter assay. PPAR α is a ligand-dependent transcription factor. Upon ligand binding, PPAR α becomes activated, forms a heterodimer with RXR, binds to the PPRE sequence in the promoter region of its target genes, and initiates their expression (Hunt et al., 2000; Keller et al., 1993). Activation of PPAR α largely induces expression of its target genes, but does not necessarily lead to an increase in its own expression, which may explain why the protein levels of PPAR α were not altered in either Hepa1c1c7 cells or HepG2 cells treated with triclosan.

Enhancement of DNA synthesis and suppression of apoptosis are two key events in rodent hepatocarcinogenesis mediated by the activation of PPAR α by peroxisome proliferators (Rodricks et al., 2010). Exposure of Hepa1c1c7 cells to triclosan led to a slight but significant increase in DNA synthesis, which is consistent with the dose-dependent increase in proliferating cell nuclear antigen labeling index in livers of CD-1 mice following a 13-week oral administration of triclosan (Trutter, 1993). In addition, the EC₅₀ value of triclosan on DNA synthesis induction (7.9 μ M) in Hepa1c1c7 cells was comparable to that obtained in mouse PPAR α reporter assay (9.9 μ M), suggesting that the effect of triclosan on DNA synthesis in Hepa1c1c7 cells is related to its activation effect on mouse PPAR α . In contrast, in HepG2 cells, triclosan led to a decrease in DNA synthesis, consistent with the decreased luciferase activity in human PPAR α reporter assays. Interestingly, several PPAR α activators, including clofibric acid, bezafibrate, ciprofibrate, nafenopin, and di-(2-ethylhexyl) phthalate, have also been reported to reduce DNA synthesis in HepG2

cells (Goll et al., 2000).

Three facts from our study suggest that the absolute level of PPAR α protein may be not the critical factor for the effect of triclosan on DNA synthesis. First, triclosan did not alter the protein level of PPAR α in either Hepa1c1c7 cells or HepG2 cells; second, overexpression of PPAR α in Hepa1c1c7 cells did not augment the triclosan-induced increase in DNA synthesis in Hepa1c1c7 cells; and third, the lower level of PPAR α in the PPAR α knocked-down HepG2 cells did not reverse the triclosan-mediated decrease in DNA synthesis in HepG2 cells.

Several studies have reported that PPAR α activators, such as clofibric acid, ciprofibrate, and nafenopin, inhibit spontaneous and TGF β -induced apoptosis in rat primary hepatocytes and the rat hepatoma Fao cell line (Bayly et al., 1994; Goll et al., 1999), but not in human primary hepatocytes (Goll et al., 1999). Similarly, our study demonstrated that triclosan, at non-cytotoxic concentrations, suppressed both basal and TGF β -induced apoptosis in mouse Hepa1c1c7 cells, but not in human HepG2 cells. These data clearly demonstrate that mouse Hepa1c1c7 cells and human HepG2 cells respond differently to triclosan.

Triclosan has been tested in several high-throughput ToxCast assays (<http://actor.epa.gov/dashboard/>), including ATG_PPAR α _TRANS, ATG_PPARE_CIS, and NVS_NR_hPPAR α , to examine the effect of triclosan on the binding and activation of human PPAR α . The ATG_PPAR α _TRANS assay utilizes HepG2 cells that express a chimeric protein that contains a GAL4 DNA binding domain, a PPAR α ligand binding domain, and a reporter transcription unit driven by GAL4 binding motif, similar to the mouse PPAR α assay described in our study. The ATG_PPARE_CIS assay utilizes HepG2 cells that express a reporter transcription unit driven by PPARE. In these two assays, triclosan was shown to have no significant effect on the activity of the reporter at concentrations ≤ 7.5 μ M following a 24 h treatment, which is consistent with our findings, while ambiguous results were obtained at higher concentration of triclosan (23 μ M). Triclosan up to 50 μ M was also shown to be inactive in the NVS_NR_hPPAR α assay,

which directly measures the binding behavior between chemicals and human PPAR α in a cell-free system. These data from ToxCast assays were consistent with our findings that triclosan is not an activator of human PPAR α .

Due to the extensive use of triclosan, the general population is exposed to triclosan through the ingestion or dermal contact with daily hygiene products and the consumption of food and drinking water contaminated with triclosan. Several studies have shown that the blood levels of total triclosan in humans following use in either mouth rinses or dentifrices vary among different individuals and can be as high as 1.4 μ M (Bagley and Lin, 2000; DeSalva et al., 1989; Sandborgh-Englund et al., 2006). Mice dermally treated with 10 and 100 mg triclosan per kg body weight have plasma levels of triclosan at 5 and 50 μ M, respectively (Fang et al., 2014). In the present study, differential effects of triclosan on the activation of mouse and human PPAR α were observed at concentrations comparable to the plasma levels in mice and approaching plasma levels found in humans.

In conclusion, we have demonstrated that triclosan behaved as an activator of PPAR α using a mouse PPAR α reporter assay, and stimulated ACOX1 expression and DNA synthesis and suppressed TGF β -induced apoptosis in mouse Hepa1c1c7 cells. In contrast, triclosan was not an activator of PPAR α in a human PPAR α reporter assay, and decreased ACOX1 expression and DNA synthesis and increased TGF β -induced apoptosis in human HepG2 cells. These data suggest that changes observed due to the activation of PPAR α in mouse liver will unlikely be the mechanism responsible for any triclosan induced alterations in human liver.

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Figure legends

Fig. 1 Effects of triclosan on the activation of mouse and human PPAR α . Reporter cells expressing hybrid mouse or human PPAR α were treated with 0.1, 0.5, 2.5, 5, 10, or 20 μ M triclosan (**A** and **B**), or co-treated with the reference activator GW590735 (20 nM) and triclosan (**C**) for 24 h. Luciferase activity was determined as described in Materials and Methods. Data are presented as the mean $\pm$ standard deviation of triplicate measurements.

Fig. 2 The half inhibitory concentration of triclosan. Hepa1c1c7 cells and HepG2 cells were exposed to 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 μ M triclosan for 24, 48, or 96 h. After the treatment, the total number of viable cells was determined by an MTT assay. The half inhibitory concentration was obtained from the cell growth curves using GraphPad Prism 6.0, and was presented as the mean $\pm$ standard deviation of three independent experiments. *, significantly ($p < 0.05$) different from Hepa1c1c7 cells at the same time point.

Fig. 3 Effects of triclosan on necrotic cell death (A), apoptosis (B), and autophagy (C) in Hepa1c1c7 cells and HepG2 cells. Cells were exposed to 0, 2.5, 5, 10, or 20 μ M triclosan for 24, 48, or 96 h. (**A**) Data from the LDH release assay were normalized by the total number of viable cells, which was determined using an MTT assay. The data are normalized to the control value at each time point. Columns and bars are means and standard deviations for three separate experiments. *, significantly ($p < 0.05$) different from the control group at the same time point. (**B**) Apoptosis was determined by caspase 3/7 activity. Etoposide (100 μ M) was used as positive control. The data are normalized to the control value at each time point. Columns and bars are means and standard deviations for three separate experiments. *, significantly ($p < 0.05$) different from the control group at the same time point. (**C**) Representative western blot analysis of the protein levels of autophagy markers in Hepa1c1c7 cells and HepG2 cells treated with triclosan for 24 h. Each protein was measured in triplicate and β -actin was used as the loading control. (**D**) Relative protein levels of autophagy markers in Hepa1c1c7 cells and

HepG2 cells treated with triclosan for 24 h. The data are normalized to the control value. Columns and bars are means and standard deviations for three separate experiments.

Fig. 4 Effects of triclosan on the protein levels of PPAR α and its target gene ACOX1 in Hepa1c1c7 and HepG2 cells. (A) Representative western blot analysis of the protein levels of PPAR α and ACOX1 in Hepa1c1c7 cells (left panel) and HepG2 cells (right panel) treated with 0, 2.5, 5, 10, or 20 μ M triclosan for 24 h. β -Actin was served as the internal reference. (B) Relative protein levels of PPAR α and ACOX1 in Hepa1c1c7 cells and HepG2 cells treated with triclosan for 24 h. The data are normalized to the control value. Columns and bars are means and standard deviations for three separate experiments. ‡, significant concentration-related trend ($p < 0.05$); *, significantly ($p < 0.05$) different from the control group.

Fig. 5 Effects of triclosan on DNA synthesis in Hepa1c1c7 cells and HepG2 cells. DNA synthesis was assessed by BrdU incorporation as described in Materials and Methods. Hepa1c1c7 cells (A and C) or HepG2 cells (B and D) were treated with 0, 2.5, 5, 10, or 20 μ M triclosan (A and B) for 24, 48, or 96 h, or the PPAR α potent activator GW7647 (0, 0.1, 1, or 10 μ M) (C and D) for 24 h. Columns and bars are means and standard deviations for three separate experiments. ‡, significant concentration-related trend ($p < 0.05$); *, significantly ($p < 0.05$) different from the control group.

Fig. 6 Effects of PPAR α expression level on triclosan-mediated alterations in DNA synthesis in Hepa1c1c7 cells and HepG2 cells

(A) Comparison of PPAR α protein levels between Hepa1c1c7/vector cells and Hepa1c1c7/PPAR α cells. Representative western blot images and relative protein levels of PPAR α are shown in upper and lower panel, respectively. (B) The knock-down efficiency of human PPAR α siRNA in HepG2 cells was determined at both the mRNA and the protein level, using real-time PCR (right panel) and western blot (left panel), respectively, at 96 h after siRNA transfection. (C) Hepa1c1c7/vector cells, Hepa1c1c7/PPAR α cells, HepG2 cells transfected with negative control siRNA, or HepG2 cells transfected with PPAR α

siRNA were treated with various concentrations of triclosan for 96 h. DNA synthesis was assessed by BrdU incorporation as described in Materials and Methods. Columns and bars are means and standard deviations for three separate experiments. *, significantly ($p < 0.05$) different from the group treated with vehicle control.

Fig. 7 Effects of triclosan on DNA damage in Hepa1c1c7 cells and HepG2 cells. DNA damage was assessed using comet assays with or without post-incubation with Fpg. H_2O_2 was used as a positive control for oxidative damage. The results shown are the mean and standard deviation of three independent experiments. *, significantly ($p < 0.05$) different from the control group; #, significantly ($p < 0.05$) different from samples without post-incubation with Fpg.

Fig. 8 Effects of triclosan on apoptosis in Hepa1c1c7 cells and HepG2 cells.

Caspase 3/7 activity in Hepa1c1c7 cells and HepG2 cells treated with 0, 2.5, 5, 10, or 20 μ M triclosan in the absence or presence of TGF β (10 ng/ml) in reduced serum conditions (0.5% FBS) for 48 h. Columns and bars are means and standard deviations for three separate experiments. *, significantly ($p < 0.05$) different from the control group; #, significantly ($p < 0.05$) different from cells treated without TGF β induction.

715 Table 1. The effects of triclosan on PPAR α activation and cytotoxicity in mouse
 716 Hepa1c1c7 cells and human HepG2 cells.

717

		Hepa1c1c7 cells	HepG2 cells
PPAR α activation	PPAR α reporter assay	Increase	Decrease
	PPAR α protein level	No effect	No effect
	ACOX1 protein level	Increase	Decrease
	DNA synthesis	Increase	Decrease
	TGF β -mediated apoptosis	Decrease	Increase
Cytotoxicity	Cell growth	Decrease	Decrease
	LDH release	Increase	Increase
	Autophagy	No effect	No effect
	Apoptosis	No effect	No effect

718

Fig. 1

Fig. 2

A

B

The concentrations of triclosan inhibiting 50% cell growth (μM)		
	Hepa1c1c7 cells	HepG2 cells
24 h	44.6 ± 4.9	66.5 ± 1.7 *
48 h	33.1 ± 1.1	30.7 ± 0.1 *
96 h	24.7 ± 1.4	26.5 ± 0.6

Fig. 3

Fig. 3 (continued)

D

Fig. 4

Fig. 5

A

B

C

D

Fig. 6

Fig. 7

Fig. 8