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Investigation of the mechanism of triclosan induced mouse liver tumors

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ABSTRACT

Chronic dietary exposure to Triclosan (TCS) produced increased incidence of liver tumors in mice. The mechanism for liver tumor induction has been attributed to activation of either peroxisome proliferator activated receptor α (PPARα) or constitutive androstane receptor (CAR). To further define the mechanism of TCS induced liver tumors, male CD-1 and C57BL/6 mice were treated with TCS at 0, 10, 100 and 200 mg/kg diet/day for 14 or 28 days. In addition, a recovery group and positive control groups for CAR or PPARα activation with either phenobarbital or diethylhexyl-phthalate were included in the 14-day study. TCS induced a dose-dependent increase in relative liver weight and centrilobular hypertrophy in both strains of mice. Hepatocyte DNA synthesis (BrdU labeling) was also increased in a dose-related pattern. In comparison with previous studies, TCS induced a significant increase in CAR/PXR (*Cyp2b10, Cyp3a11*) and PPARα (*Cyp4a10*) responsive genes in both CD-1 and C57BL/6 mice. The corresponding enzyme activity for CAR (7-pentoxyresorufin-O-dealkylase) and PPARα (peroxisomal Acyl-CoA oxidase) were also significantly increased in a similar fashion. Oxidative stress related genes *Gpx1* and *Aox1* were increased in the C57BL/6 but not in CD-1 mice. The increases in gene expression and enzyme activities returned to control levels after 14-day recovery. The present results demonstrate that both CAR and PPARα activation are involved in the TCS induced mouse liver tumor.

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1. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxy-diphenyl ether or 5-chloro-2-(2,4-dichlorophenoxy)phenol: TCS) is an antibacterial and antifungal agent used in consumer products such as antibacterial soaps, toothpaste, and cosmetics. It was first introduced more than 50 years ago (Campbell and Zirwas, 2006). In the United States, TCS is regulated by both the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA). The concentration of TCS in consumer products is usually in the range of 0.1%–0.3% (Dhillon et al., 2015), but can be up to 1%–2% in health care and clinical settings (Coia et al., 2006). Owing to its widely use and release into the environment, TCS and its degradation products have been detected in different environment matrix including water (Bedoux et al., 2012; Dhillon et al., 2015; Venkatesan et al., 2012).

Humans, as a consequence of environmental and consumer product use are exposed to TCS. Biomonitoring studies in humans have detected TCS in urine, plasma and milk (Allmyr et al., 2006; Calafat et al., 2008; Dayan, 2007; Hines et al., 2015), and even in nails (Yin et al., 2016). Experimental studies have linked TCS exposure to disruption of thyroid function (Axelstad et al., 2013; Crofton et al., 2007; Zorrilla et al., 2009), impaired muscle function (Cherednichenko et al., 2012), enhanced skin allergy (Bertelsen et al., 2013), altered estrogenic activity (Gee et al., 2008), suppression of male reproduction (Kumar et al., 2009), and spontaneous abortion through a decline of estrogen sulfotransferase activity (Wang et al., 2015a). Recent reports have also suggested that TCS may also adversely affect human immune function (Anderson et al., 2016; Barros et al., 2010; Clayton et al., 2011). Although studies have not associated TCS exposure with human

Abbreviations: ACO, peroxisomal Acyl-CoA oxidase; AhR, aryl hydrocarbon receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Aox1, Aldehyde oxidase 1; BrdU, 5-bromo-2'-deoxyuridine; DEHP, bis(2-ethylhexyl) phthalate; EPA, Environmental Protection Agency; EROD, ethoxyresorufin-O-deethylase; FDA, Food and Drug Administration; MDA, malondialdehyde; MOA, mode of action; PB, phenobarbital; PPARα, peroxisome proliferator-activated receptor alpha; PROD, pentoxyresorufin-O-dealkylase; PXR, pregnane X receptor; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance.

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cancer formation, Rodricks et al. noted "when evaluated in chronic oncogenicity studies in mice, rats and hamsters, treatment-related tumors were found only in the liver of male and female mice" (Rodricks et al., 2010) (Table 1). Therefore, only the mouse liver was a target for the tumorgenicity of triclosan.

The mechanism by which chemicals induce liver tumors in rodents has been extensively studied. Rodent liver tumors may be induced through either genotoxic (direct DNA reactive) or non-genotoxic mechanisms (non DNA reactive) (Cohen, 2010; Holsapple et al., 2006; Klaunig, 2013; Wang et al., 2015b). Non-genotoxic mechanisms can be further defined into receptor mediated or non-receptor mediated pathways including cytotoxicity, oxidative stress, infectious agents. Receptor-mediated modes of action, although involving different receptors, function though the gene expression induction of an increase in cell proliferation and increase DNA synthesis that allows for the selective clonal expansion of preneoplastic hepatocytes. Agents that produce tumors in the liver through cytotoxic mechanisms induce significant hepatic necrosis that results in chronic compensatory hyperplasia. This chronic hyperplasia either induces new initiated hepatocytes through misrepair of DNA and/or allows for the selective clonal expansion of preneoplastic hepatocytes already present (Klaunig, 2013). Oxidative stress is seen with inflammation as well as during the metabolism of xenobiotics. Oxidative stress may also contribute to other modes of action as an associative factor (Corton et al., 2014). Infectious agents similarly may induce liver cancer via consistent damage and injury, having a cytotoxic component to the mechanism of action.

TCS has not been shown to be mutagenic or genotoxic in standard bioassays. As such the mechanism of action for TCS induced mouse liver tumorigenesis has been previously attributed to be through non-genotoxic mechanisms by activation of peroxisome proliferator activated receptor alpha (PPARα) (Rodricks et al., 2010). In contrast, a recent study in C57BL/6 mice reported that TCS activated the nuclear receptor constitutive androstane receptor (CAR) whereas having no significant effect on PPARα (Yueh et al., 2014). Yueh et al. also reported that TCS treatment promoted mouse liver tumor development using an initiation-promotion protocol. However, this study failed to demonstrate that TCS-induced mouse liver tumors was exclusively CAR dependent since CAR deficient mice ($Car^{-/-}$) mice also had significantly higher tumors than non-TCS treated control (C57BL/6 mice), though the tumor number was only about half of that seen in $Car^{+/-}$ mice (Yueh et al., 2014). These data indicated that CAR activation alone was not sufficient for the development of liver tumors.

In an effort to resolve the discrepancies of CAR versus PPARα mediated processes in TCS induced liver tumors in mice, the current study was performed. We examined the accepted possible modes of action of rodent carcinogenic agents in the liver including receptor-mediated processes, cytotoxicity, oxidative stress and inflammation by TCS using both the CD-1 (from the chronic bioassay) and the C57BL/6 mice (from the Yueh et al. (2014) study).

Table 1
Liver tumor incidence in CD-1 mice following chronic treatment with triclosan in diet (taken from Rodricks et al., 2010).

Number of Tumor Bearing mice[a,b]						
Dose level (mg/kg/day)	Adenoma		Carcinoma		Adenomas and carcinomas combined	
	Males	Females	Males	Females	Males	Females
0	5	0	2	0	6	0
10	7	1	3	0	10	1
30	13*	3*	6	1	17**	3*
100	22**	6**	11**	1	32**	6**
200	26**	11**	24**	14**	42**	20**

**$p \leq 0.01$; *$p \leq 0.05$.
[a] Numbers represent tumor-bearing mice in each group.
[b] Studies involved 60 mice per sex, per dose group.

2. Materials and methods

2.1. Chemicals and reagents

Triclosan (5-Chloro-2-(2,4-dichlorophenoxy)phenol or TCS, ≥97% purity, CAS 3380-34-5) was purchased from EMD Millipore (Cat# 647950, Lot# 00000, Billerica, MA). Phenobarbital Sodium (PB, Cat# P5178) and Bis(2-ethylhexyl) phthalate (DEHP or Dioctyl phthalate, ≥99.5% purity, Cat# D201154) were purchased from Sigma-Aldrich (St Louis, MO). ALT/AST reagent kits were purchased from Fisher Scientific (Waltham, MA). Anti-BrdU antibody was obtained from Biogenex (San Ramon, CA). All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

2.2. Animals and treatment

Male CD-1 and C57BL/6 mice (5–7 weeks old) were purchased from Charles River Laboratories (Wilmington MA). Mice were housed in the AAALAC certified animal facility at Indiana University, Bloomington, Indiana. All animals were maintained in accordance to the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Research (NRC, 2011) and treatment protocols were approved by the Institutional Animal Care and Use Committee at Indiana University. Mice were housed (five per cage) in individually ventilated cages under conditions of controlled temperature (22 ± 1 °C), humidity (40–70%), and light cycle (12 h/12 h), and were given food and municipal water ad libitum. All animals were acclimated for 7 days prior to the initiation of the study. Individual mice were ear-tagged for identification throughout the study and randomly assigned to control or treatment groups.

A total of 110 male mice (55 CD-1 and 55 C57BL/6 mice) were used in the studies. Mice (five/group) of both strains were treated with TCS at doses of 0 (control), 10, 100, or 200 mg/kg diet/day for 14 days or 28 days. Concurrent positive control groups including PB (150 mg/kg diet/day) DEHP (1200 mg/kg diet/day) were also performed 14 days. In a recovery study was simultaneously performed in which mice (five from each strain) were treated with TCS at 200 mg/kg diet/day for 14 days and then given control diet for an additional 14 days.

Seven days prior to each scheduled sacrifice, mice were given bromodeoxyuridine (BrdU, 8 g/L) in drinking, supplemented with 1% glucose for palatability, for subsequent measurement of DNA synthesis. BrdU containing water was prepared freshly and changed twice weekly. The body weight of each animal was recorded weekly and at terminal sacrifice. Mice were sacrificed after 14 or 28 days of treatment by CO_2 asphyxiation in accordance with animal care procedures. Blood was collected via cardiac puncture from each animal for serum hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement. The liver was removed *in toto* and weighed. A portion of the liver was fixed in 10% neutral buffered formalin for histopathology analysis by hematoxylin and eosin (H&E) staining and immunohistochemistry staining with anti-BrdU (Biogenex Laboratories, San Ramon, CA). The rest of the liver was snap frozen in liquid nitrogen and stored at −80 °C.

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