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Administration of low dose triclosan to pregnant ewes results in placental uptake and reduced estradiol sulfotransferase activity in fetal liver and placenta

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ABSTRACT

Sulfonation is a major pathway of estrogen biotransformation with a role in regulating estrogen homeostasis in humans and sheep. Previous *in vitro* studies found that triclosan is an especially potent competitive inhibitor of ovine placental estrogen sulfotransferase, with K_{ic} of < 0.1 nM. As the placenta is the main organ responsible for estrogen synthesis in pregnancy in both women and sheep, and the liver is another site of estrogen biotransformation, this study examined the effects of triclosan exposure of pregnant ewes on placental and hepatic sulfotransferase activity. Triclosan, 0.1 mg/kg/day, or saline vehicle was administered to late gestation fetal sheep for two days either by direct infusion into the fetal circulation or infusion into the maternal blood. On the third day, fetal liver and placenta were harvested and analyzed for triclosan and for cytosolic estradiol sulfotransferase activity. Placenta contained higher concentrations of triclosan than liver in each individual sheep in both treatment groups. There was a negative correlation between triclosan tissue concentration (pmol/g tissue) and cytosolic sulfotransferase activity (pmol/min/mg protein) towards estradiol. These findings demonstrated that in the sheep exposed to very low concentrations of triclosan, this substance is taken up into placenta and reduces estrogen sulfonation.

1. Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy) phenol (Fig. 1), a halogenated diphenyl ether, is an antibacterial agent that was added to a number of personal care products including hand soaps, toothpastes, mouthwashes, bandages and clothing (Yueh and Tukey, 2016). As a result of its widespread use, triclosan has been found in wastewater, sewage treatment plants and sewage sludge around the world (Bester, 2005; Dhillon et al., 2015; Fernandes et al., 2011; Hua et al., 2005). The main routes of human exposure are thought to be buccal absorption, ingestion and dermal absorption (Bagley and Lin, 2000; Lin, 2000). Studies have documented the presence of triclosan in between 50 and 100% of human blood and urine samples, depending on the population studied (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Arbuckle et al., 2015a; Arbuckle et al., 2015b; Calafat et al., 2009; Calafat et al., 2008; Geens et al., 2012; Liu et al., 2014; Philippat et al., 2013; Provencher et al., 2014; Pycke et al., 2014; Sandborgh-Englund et al., 2006; Velez et al., 2018; Weiss et al., 2015; Woodruff et al., 2011). Triclosan has also been measured in autopsy samples of liver, brain and adipose tissue (Geens et al., 2012).

The antibacterial properties of triclosan derive from its potent inhibition of the enoyl-acyl carrier protein reductase, an enzyme essential in fatty acid synthesis in bacteria (Levy et al., 1999; McMurry et al., 1998). By blocking the active site of the enzyme, triclosan prevents the bacteria from synthesizing fatty acid, which is necessary for reproducing and building cell membranes. Humans do not express this enzyme, so arguments were made that triclosan was unlikely to pose a risk to people (Dayan, 2007; Jones et al., 2000; Rodricks et al., 2010).

It has been found that triclosan exhibits biological activities other than its antibacterial function. Potential health concerns related to the use of triclosan include endocrine disruption, effects on drug- and hormone-metabolizing enzymes, the potential for development of antibiotic resistance, and the formation of carcinogenic by-products during triclosan degradation (Crofton et al., 2007; Gee et al., 2008; Levy, 2000, 2001; Schweizer, 2001; Stoker et al., 2010; Veldhoen et al., 2006; Wang et al., 2004; Zorrilla et al., 2009). Administered to pregnant sheep, triclosan was found to alter transcriptomics in the fetal hypothalamus (Rabaglino et al., 2016). Triclosan directly inhibits and indirectly induces certain phase II UDP-glucuronosyltransferase (UGT) and PAPS-sulfotransferase (SULT) enzymes (James et al., 2010; Wang

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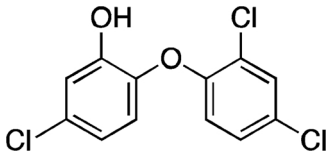

Fig. 1. Structure of triclosan.

et al., 2004; Wang and James, 2006; Zorrilla et al., 2009). Administration of triclosan (0–1000 mg/kg) to rats for 4 days induced dose-dependent increases in *Ugt1a1* and *Sult1c1* mRNA expression, and increased liver microsomal glucuronidation activity toward thyroxine, but did not affect mRNA expression of *Ugt1a6 or 2b5 and* reduced mRNA expression of *Sult1b1* (Paul et al., 2010). It was shown that triclosan serves as a substrate and inhibitor of human hepatic glucuronidation and sulfonation detoxification pathways with drug and xenobiotic substrates (Wang et al., 2004). Triclosan was found to be an exquisitely potent competitive inhibitor of sheep placental estrogen sulfotransferase activity toward 17β-estradiol with a K_{ic} value of 0.09 nM (James et al., 2010). Triclosan also inhibited estrone sulfonation, exhibiting an IC_{50} value of 0.6 nM (James et al., 2010).

Sulfoconjugation is an essential reaction in the phase II biotransformation of various endogenous and foreign substances, including drugs, toxic chemicals, steroid hormones, and neurotransmitters (Strott, 2002). Sulfonation plays a vital role in the transport of steroids. Natural estrogens are steroid hormones that, while present in both males and females, are usually present at considerably higher levels in females of reproductive age. The steroid sex hormone, 17β-estradiol (E2) is one of the three main estrogens naturally produced in the body. E2 has two hydroxyl groups in its molecular structure, while estrone has one (E1) and estriol has three (E3). The sulfated, inactive forms of E1 and dehydroepiandrosterone (DHEA) are the primary "transport forms" of steroids and serve as the precursors of androgen and estrogen biosynthesis (Hobkirk, 1985; Morato et al., 1965; Mortola and Yen, 1990). Estrogen sulfotransferase, SULT1E1, is the major isoform responsible for E1 and E2 sulfonation at physiological concentrations (Zhang et al., 1998), though other SULTs can catalyze estrogen sulfonation at lower efficiencies (Wang and James, 2005).

The pregnant sheep has been used extensively as a model to study physiology in pregnancy, in particular pathways of estrogen homeostasis (Purinton and Wood, 2000; Wood, 2005; Wood et al., 2003). In this study, pregnant ewes and their fetuses were administered an environmentally relevant dose of triclosan by infusion and the effects on estrogen sulfotransferase investigated.

2. Materials and methods

2.1. Chemicals

[3 H]-17β-estradiol (E2), > 97% pure, 60 Ci/mmol, was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). Triclosan used for sheep dosing was purchased from TCI America (Portland, Oregon) and shown to be > 99.8% pure by reverse-phase HPLC analysis with UV detection at 280 nm. Triclosan sulfate was purchased from Toronto Research Chemicals, North York, Ontario. The internal standard for LC/MS/MS analysis was 2,2′,5′-trichloro-biphenyl-4-ol (4′-OH-CB18), purchased from Accustandard (New Haven, CT). Water utilized in these experiments was purified by Milli-Q water system to 18 MΩ. *Helix pomatia* beta-glucuronidase containing sulfatase activity (G0751) was obtained from Sigma-Aldrich, St. Louis, MO. Molecular weight standards, 12% mini-Protean TGX gels and nitrocellulose membranes were purchased from BioRad, Hercules, CA. All other chemicals and solvents were of the highest grade available and obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

2.2. Animal treatment

Experiments were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the American Physiological Society's Guiding Principles for Research Involving Animals and Human Beings. The administered triclosan dose was calculated to be comparable to exposure of an adult to personal care products such as toothpaste containing 0.3% triclosan. Time-dated pregnant ewes between 120 and 130 days gestation were administered 0.1 mg triclosan/kg/day for 2 days either through direct infusion into the fetal circulation or through administration to the ewe. On the third day, hepatic and placental tissues were harvested from the late gestation fetuses. Other tissues from these animals were examined for genomic effects of triclosan and a full description of the exposure protocol has been published (Rabaglino et al., 2016). Briefly, for direct exposure, chronically-catheterized fetal sheep were intravenously infused with vehicle (dimethylsulfoxide:saline, 1:1) or triclosan solution, whereas indirect fetal exposure was achieved through intravenous infusion of vehicle or triclosan into the maternal circulation (Rabaglino et al., 2016). Eight fetuses being carried by six ewes received triclosan directly, and maternal exposure was to three ewes carrying five fetuses. After infusion was complete, the pregnant ewes and fetuses were humanely euthanized with an overdose of sodium phenobarbital. Upon confirmation of cardiac arrest, fetal tissues were rapidly removed and snap frozen in liquid nitrogen. Tissue samples were stored at −80 °C until use in these experiments. Portions of the tissue samples were analyzed for triclosan or processed into subcellular fractions for measurement of enzyme activity.

2.3. Preparation of subcellular fractions

Subsamples of liver and placental cotyledon from individual sheep, 0.2 to 1.5 g, were homogenized in 4 volumes of homogenizing buffer containing 0.25 M sucrose, 0.05 M Tris base, pH 7.4, 5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged as described in previous work to separate microsomes and cytosol fractions (James et al., 2010). Protein concentrations in the subcellular fractions were determined by the bicinchoninic acid method, with bovine serum albumin as a standard (Thermo, Rockford, IL).

2.4. Analysis of triclosan in tissues

Triclosan concentrations in liver and placenta were measured by LC/MS/MS. All glassware was rinsed with acetone and deionized water before use. We maintained a triclosan-free area of the laboratory for all analytical work, to avoid accidental contamination of samples. Samples of all tissues were analyzed for total triclosan following hydrolysis of tissue homogenates with glucuronidase/sulfatase and sample cleanup. Duplicate tissue samples, 0.5 g, were homogenized with a bio-homogenizer (Biospec products, Inc., Bartlesville, OK) in 1.5 ml 0.1 M ammonium acetate buffer pH 5 and transferred to a glass tube. The vessel was rinsed with 2.5 ml buffer that was added to the tube together with 0.025 ml of a 0.6 μM methanol solution of 4′-OH-CB18 as internal standard. To this was added 1 ml of *Helix pomatia* β-glucuronidase containing sulfatase (15,000 units) and the mixture was incubated for 24 h at 37 °C. Additional duplicate samples from fetal liver and placenta of fetally-infused sheep were analyzed without hydrolysis, and these samples were taken to the next step without incubation. Preliminary studies with biosynthesized triclosan sulfate and triclosan glucuronide standards demonstrated that hydrolysis of these conjugates to triclosan was not complete before 24 h. After hydrolysis, acetonitrile containing 1% formic acid, 2 ml, was added to each tube and vortex-mixed. The tube was centrifuged at 3000 g for 10 min and the supernatant transferred to a clean tube. The pellet was extracted twice more and the supernatants pooled. The pooled supernatants were centrifuged again