Effects of tris(2,3-dibromopropyl) isocyanurate on steroidogenesis in H295R cells

Xun Li¹ · Yu Pan¹ · Chang Wang² · Minjie Chen¹ · Yuchen Liu¹ · Jia Li¹ · Zhen Zhou³ · Jinhua Xu¹ · Yong Liang^{1,2} · Maoyong Song^{2,4}

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Abstract Tris(2,3-dibromopropyl) isocyanurate (TBC) is widely used in polymer products. It is ubiquitously found in environmental matrices. Previous studies have shown that TBC has potential endocrine-disrupting effects on aquatic organisms. However, the effects on adrenocortical function and the underlying mechanisms are not well characterized. In present study, the H295R cell line was employed to investigate the potential endocrine-disrupting action of TBC. TBC inhibited basal and forskolin-induced sex hormone production but did not exhibit cytotoxicity. The expression of the steroidogenic genes, *StAR*, *3 β HSD2*, *CYP17*, *CYP21*, *CYP19*, *HMGR*, *17 β HSD1*, *17 β HSD4* and *CYP11A*, was not changed upon TBC exposure. However, the cAMP inducer forskolin strongly induced the expression of all these genes, except for *HMGR*, *17 β HSD1* and *17 β HSD4*. TBC blocked forskolin-induced *StAR*, *3 β HSD2*, *CYP17*, *CYP21* and *CYP19* mRNA expression. Our results indicate that TBC can inhibit sex hormone biosynthesis due

to the interference with steroidogenic gene transcription in activated condition.

Keywords Tris(2,3-dibromopropyl) isocyanurate · H295R · Sex hormone biosynthesis · Steroidogenic gene · Endocrine-disrupting action

Introduction

Brominated flame retardants (BFRs) are widely used in a large variety of polymer products with the purpose of protecting materials against ignition and reducing fire-related deaths and injuries. However, the major commercial BFRs, including polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD), are ubiquitous in biotic and abiotic samples (de Wit et al. 2006; Hale et al. 2006; Law et al. 2006; Wang et al. 2009; Wu et al. 2012; Zhu et al. 2012). There are compelling evidences that some BFRs can cause neurotoxicity, immunotoxicity and endocrine toxicity (Birnbbaum and Staskal 2004; Costa and Giordano 2007; Guo et al. 2015; Legler and Brouwer 2003; Li et al. 2011b; Molnár et al. 2015; Yin et al. 2015). Thus, the use of certain BFRs is already forbidden or restricted in many developed countries. Consequently, the demand for some alternative to the toxic BFRs, such as tris(2,3-dibromopropyl) isocyanurate (TBC, CAS No. 52434-90-9), has further increased (Ruan et al. 2009). The annual production volume of TBC was more than 500 metric tons in China (Ruan et al. 2009). As the increasing market demand for electronic and textile products on worldwide, the production of TBC continuously increases.

TBC was firstly found in environmental samples near a manufacturing plant in Hunan Province of China in 2009.

✉ Yong Liang
ly76@263.net

✉ Maoyong Song
smsong@rcees.ac.cn

¹ School of Medicine, Jiangnan University, Wuhan 430056, People's Republic of China

² Institute of Environment and Health, Jiangnan University, Wuhan 430056, People's Republic of China

³ Key Laboratory of Optoelectronic Chemical Materials and Devices, Ministry of Education, School of Chemical and Environmental Engineering, Jiangnan University, Wuhan 430056, People's Republic of China

⁴ State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, People's Republic of China

Higher concentrations of TBC were detected in river water (2.33–163 ng/L), surface sediments (85.0–6029 ng/g), soils (19.6–672 ng/g), earthworms (9.75–78.8 ng/g) and carp (12.0–646 ng/g) (Ruan et al. 2009). Meanwhile, TBC was detected in 77 % of 11 species of mollusks collected around the Bohai Gulf, and the concentration on a dry weight (DW) basis was up to 12.1 ng/g (Zhu et al. 2012). Moreover, TBC was also found in all farm soils at a region in southeast Beijing in 2011, compared to only 25 % at some region in 2010. The concentration ranges from below detection limit to 1.62 ng/g (DW) in farm soil samples (Wang et al. 2013). According to these data, *in vivo* studies have been performed to evaluate the toxicity of TBC. They found that TBC cause swim bladder inflation failure in zebrafish larvae (Li et al. 2011a), the impaired cognition and provoked depression-like behaviors in adult rats (Ye et al. 2015) and induced liver and lung damage in mice (Li et al. 2015). *In vitro* study indicated that TBC might be one of the causative compounds inducing neuronal toxicity in the environmental samples collected near a TBC production facility (Qu et al. 2011). Also, TBC has been shown to remarkably inhibit estrogen-dependent vitellogenin (VTG) gene expression in liver and further affect gonadal development in both male and female zebrafish (Zhang et al. 2011). Therefore, these initial results indicate that TBC may have endocrine-disruptive effects and interference with the hormonal balance, resulting in developmental and reproductive abnormalities. Because steroid hormones can be synthesized in gonads and adrenal glands, we sought to determine whether TBC affected steroid hormone synthesis in adrenal glands.

The mammalian adrenal glands belonged to the endocrine system site on top of the kidneys. Each adrenal gland is divided into two distinct structures, the adrenal cortex and the medulla. The medulla secretes catecholamines. The adrenal cortex contains three concentric zones of steroid-synthesizing cells synthesizing and secreting mineralocorticoids such as aldosterone, glucocorticoids such as cortisol, androgens such as testosterone, estrogens such as estradiol, which are derived from cholesterol. These steroids play essential roles in the regulation of reproduction in vertebrates and also regulate a number of processes related to development and growth, being involved in sexual differentiation, the secondary sex characteristics and sexual behavior patterns (Ruiz-Cortés 2012). Because of containing large stores of lipophilic agents, the adrenal cortex is more sensitively assaulted by chemicals than the medulla. Chemicals inhibit the normal function of the adrenal cortex, resulting in impaired steroidogenesis (Rosol et al. 2001). In order to understand the molecular mechanisms by which some compounds affect steroidogenesis, well-characterized experimental models are required. A useful *in vitro* model for researches on steroidogenesis is

the H295R cell line (Gazdar et al. 1990; Rainey et al. 1994; Rehman et al. 2003). The H295R adrenocortical cell line is pluripotent cell line, derived from an adrenocortical carcinoma. H295R cells can produce all of the steroid hormones in three different regions of human adrenal cortex and are capable of expressing the key enzyme (aromatase) in the process of steroid synthesis (Wang and Rainey 2012). The H295R steroidogenesis assay has been approved by the Environmental Protection Agency for assessing effects of chemical agents on steroidogenesis (Gracia et al. 2006). Besides, the H295R cells treated with synthetic cAMP analogues or activation of adenylate cyclase by forskolin could make the steroidogenic transcript patterns similar to those in normal human adrenal cells, suggesting that the H295R cell line can serve as a good model to assess the risk of adrenocortical toxicity (Cobb et al. 1996; Li et al. 2013; Oskarsson et al. 2006).

In order to investigate the effects of TBC on steroidogenic pathways and adrenocortical toxicity, we exposed H295R cells to TBC at different concentrations in the absence and presence of 10 μ M forskolin for 72 h. Production of sex steroid hormones (testosterone and 17 β -estradiol) and transcription of nine genes encoding the steroidogenic enzymes (HMGR, StAR, CYP11A, 3 β HSD2, CYP17, CYP21, 17 β HSD1, 17 β HSD4 and CYP19) were investigated.

Materials and methods

Chemicals

Tris(2,3-dibromopropyl) isocyanurate (TBC, 97 % purity) was purchased from Sigma-Aldrich (St Louis, MO). Stock solutions (1 M) were prepared by dissolving TBC in dimethyl sulfoxide (DMSO, AMRESCO) and stored at 4 °C. All the chemicals used in this study were analytical grade.

Cell culture

The H295R cells (CRL-2128) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM/F-12 (HyClone, SH30023.01) supplemented with 1 % insulin–transferrin–selenium G (ITS-G, Gibco BRLm, 41400-045), 1 % penicillin–streptomycin (Gibco BRL, 15140-122) and 2.5 % Nu-Serum (BD Bioscience, 35500). The cells were maintained at 37 °C containing 95 % air and 5 % CO₂ atmosphere. The culture medium was refreshed two or three times a week. Cells were cultured for a minimum of five passages using new NCI-H295R batches prior to exposure to the tested chemical.

Experimental design

TBC was dissolved in DMSO as a stock solution, and the exposure and control groups were received 0.1 % DMSO. For cell viability measurements, exponentially growing cells were seeded at a density of 1×10^4 cells in 200 μ L of culture medium in a 96-well culture plate. After 24 h, cells were exposed to different concentrations of TBC and the medium was refreshed with steroid-free medium (phenol red-free DMEM/F-12 medium (HyClone, SH30272.01), 1 % ITS-G, 2.5 % Nu-Serum, 100 U/mL streptomycin–penicillin). After 72 h of exposure, cell morphology was observed by light microscopy.

For the measurement of hormone and gene transcription, the cells were grown at a density of 3×10^5 cells/mL in 2 mL vol/well in 24-well plates. To minimize the basal estrogenic effects of growth media on cells, they were incubated in steroid-free medium for 24 h before chemical treatment. Ten micromolar forskolin was added to mimic the ACTH upsurge according to previous studies (Furuta et al. 2008; Li and Wang 2005). The culture medium was removed after 24 h, and cells were treated with different concentrations of the tested TBC in the absence and presence of 10 μ M forskolin for 72 h. Cells treated with only DMSO (0.1 %) were set as solvent control (SC). At the end of exposure, the medium was collected for testosterone and 17 β -estradiol analysis. After removal of the medium, cells were lysed by the addition of 1 mL well⁻¹ of TRIzol reagent (Invitrogen, 15596018) for total RNA isolation. All samples were stored at -80°C prior to analysis.

Hormone measurement

The culture medium was centrifuged at 3000 r/min for 10 min to remove dead cells and cell debris before analysis. T and E2 were measured using chemiluminescent microparticle immunoassay technology in Abbott AxSYM System Automated Immunoassay Analyzer (Abbott Diagnostics, Abbott Park, Illinois, USA) according to the method described by Wang et al. (2014).

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent following the manufacturer's protocol. The purity of the total RNA was measured by a UV spectrophotometer (BioPhotometer Plus, Eppendorf, Hamburg, Germany). The quality and integrity were verified by measuring the 260/280 nm ratio and by electrophoresis on a 1 % agarose formaldehyde gel. In this study, 2 μ g of total RNA was reverse-transcribed to cDNA by MMLV reverse transcriptase according to the

manufacturer's instructions. The primer sequences are shown in Table 1. Each PCR mixture in a total volume of 20 μ L contained 1 μ L of cDNA template, 0.1 μ M primers, Milli-Q water and 10 μ L of 2 $\times$ SYBR QPCR Master Mix. The thermal cycling program included an initial denaturing step at 94°C for 8 min, followed by 35–45 cycles of denaturing (95°C for 20–30 s), primer annealing (at 50 – 64°C for 40–60 s) and cDNA extension (68 – 72°C for 20–40 s), and then a final cycle of 94°C for 15–40 s, 50 – 60°C for 30–60 s and 72°C for 5–10 min. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired products and any primer–dimers or DNA contaminants. Specific details of the assay parameters such as primers used and annealing temperatures were derived from a previous study (Hilscherova et al. 2004). Relative expression of mRNA was quantified by the comparative C_T method described previously (Ji et al. 2010; Livak and Schmittgen 2001).

Statistical analysis

Except when indicated, the data are shown as the mean $\pm$ SD of three independent experiments ($n = 3$), with each data point within an independent experiment determined in duplicate or triplicate. Nonparametric statistical analysis was performed by a Mann–Whitney test because of non-normal distribution for these data. Statistical analysis was performed using SPSS 13.0. For all experiments, $P \leq 0.05$ was considered as statistically significant.

Results

Effect of TBC on H295R cells viability

Preliminary experiments based on visual inspection under a microscope showed that no cytotoxicity was observed at currently tested concentrations (1×10^{-9} to 1×10^{-5} M) of TBC. This dose range is used for the following experiments to estimate TBC effects on steroidogenesis without causing cytotoxicity.

Effects of TBC on hormone production

The basal amount of E2 and T in vehicle-treated cells was 299 and 599 pg/mL, respectively. After forskolin stimulation, the amount of E2 and T increased to 885 and 1100 pg/mL, respectively (Fig. 1). TBC exposure reduced E2 production in a dose-dependent manner. In addition, 1×10^{-6} M TBC significantly decreased the forskolin-induced secretion of E2 and T (Fig. 1).

Table 1 Primer sequences for genes analyzed

Gene	Sense primer	Antisense primer_accession number
<i>β-Actin</i>	CACTCTTCCAGCCTTCCTTCC	AGGTCTTTGCGGATGTCCACNM_001101
<i>CYP11A</i>	GAGATGGCACGCAACCTGAAG	CTTAGTGTCTCCTTGATGCTGGCNM_000781
<i>CYP11B2</i>	TCCAGGTGTGTTTCAGTAGTTCC	GAAGCCATCTCTGAGGTCTGTGNM_000498
<i>CYP17</i>	AGCCGCACACCAACTATCAG	TCACCGATGCTGGAGTCAACNM_000102
<i>CYP19</i>	AGGTGCTATTGGTCATCTGCTC	TGGTGAATCGGGTCTTTATGGNM_000103
<i>CYP21</i>	CGTGGTGCTGACCCGACTG	GGCTGCATCTTGAGGATGACACNM_000500
<i>3βHSD2</i>	TGCCAGTCTTCATCTACACCAG	TTCCAGAGGCTCTTCTTCGTGNM_000198
<i>17βHSD1</i>	CTCCCTCTGACCAGCAACC	TGTGTCTCCCACGCAATCTCNM_000413
<i>17βHSD4</i>	TGCGGGATCACGGATGACTC	GCCACCATTCCTCACAACCTCNM_000414
<i>HMGR</i>	TGCTTGCCGAGCCTAATGAAAG	AGAGCGTTCGTGGGTCCATCNM_000859
<i>StAR</i>	GTCCACCCCTGCCTCTGAAG	CATACTCTAAACACGAACCCACCNM_000349

Fig. 1 Effects of TBC on hormone production in H295R cells. Cells were treated with 0.1 % DMSO (vehicle control), 10 μM forskolin (For; positive control), 100 nM–100 μM TBC or TBC + Forskolin for 72 h. Results are expressed as the mean ± SD of triplicate measurements in three replicate samples. *a* Response significantly different than the vehicle control ($P \leq 0.05$); *b* response significantly different than forskolin alone ($P \leq 0.05$)

Effects of TBC on expression of steroidogenic genes

A comparison of the vehicle control groups showed that stimulation with forskolin led to significant changes in expression of all tested genes (Fig. 2). All genes, except for *HMGR* and *17βHSD1*, were up-regulated. The up-regulation was most evident for *StAR*, *CYP11A*, *3βHSD2*, *CYP17*, *CYP19* and *CYP21*, with fold changes of 2.86, 3.05, 13.48, 5.04, 11.52 and 5.02, respectively. TBC at all tested concentrations did not affect the basal or forskolin-induced expression level of *HMGR*, *CYP11A*, *17βHSD1* and *17βHSD4*. The gene expression induced by forskolin of *StAR* and *3βHSD2* was significantly inhibited by TBC at 1×10^{-6} and 1×10^{-5} M. The forskolin-induced *CYP17* gene expression was significantly inhibited by TBC at 1×10^{-5} M. For genes *CYP21* and *CYP19*, forskolin-induced gene expression was also inhibited by TBC in a dose-dependent manner (Fig. 2). The alterations in steroidogenic gene expression and hormone production after TBC exposure are summarized in Fig. 3.

Discussion

TBC, as a new emerging pollutant, is used as additive substance in many consumer products. Due to its widespread distribution and non-biodegradable persistent in the environment, the increasing exposure of TBC to human and wildlife has drawn much attention. Although the endocrine-disrupting effects of TBC have been studied, little is known about the TBC effects on the adrenal cortex steroidogenesis. In this study, we used in vitro model to examine the effects of TBC on steroidogenesis and the probable mechanisms, to provide a scientific evidence for the government to control its use.

TBC may alter adrenal cortex function through modulating steroidogenic pathways. H295R cells were used to research how TBC disrupted steroidogenesis in the present study. We exposed H295R cells to different concentrations of TBC in the absence or presence of 10 μM forskolin for 72 h, and then the concentrations of two steroid hormones, E2 and T, in the culture medium supernatant and the relative expression level of nine steroidogenic-related genes

Fig. 2 Effects of TBC on the expression of steroidogenic genes in H295R cells. Cells were treated with 0.1 % DMSO (vehicle control), 10 μ M forskolin (For; positive control), 1 nM–10 μ M TBC or TBC + forskolin for 72 h. Results are expressed as the mean $\pm$ SD of triplicate measurements in three replicate samples. *a* Response significantly different than the vehicle control ($P \leq 0.05$); *b* response significantly different than forskolin alone ($P \leq 0.05$)

(*StAR*, *3 β HSD2*, *CYP17*, *CYP21*, *CYP19*, *HMGR*, *17 β HSD1*, *17 β HSD4* and *CYP11A*) were measured. Our results showed that forskolin treatment of the H295R cells resulted in more hormone secretion and a generally increase expression of steroidogenic-related genes, which confirms its capability for inducing steroidogenesis in H295R cells and is consistent with previous reports (Gracia et al. 2006; Hecker et al. 2006). Additionally, TBC exposure caused significantly decrease in steroid hormones in H295R cells. This effect was most obvious in the agonist-treated cells. The results are summarized in Fig. 3 showing that TBC affected the expression levels of some genes associated with the steroidogenic pathway and the reduction of two steroid hormones. Significantly down-regulated expression of *StAR*, *3 β HSD2*, *CYP17*, *CYP21* and *CYP19* was observed after TBC treatment in this study. The *HMGR*, *17 β HSD1*, *17 β HSD4* and *CYP11A* expression was not obviously altered (Fig. 2).

HMGR and *CYP11A1* expressions are rate-limiting steps in steroidogenic pathway (Stocco and Clark 1996). Transcription levels of *HMGR* and *CYP11A1* were not significantly altered upon exposure to TBC. The relatively constant transcription expression of these genes was also

found in previous studies (He et al. 2008; Li et al. 2013; Zhang et al. 2005). Although *HMGR* and *CYP11A1* mRNA levels were not changed in this study, posttranslational modifications, such as ubiquitination, could decrease the amount of active enzyme, resulting in less of precursor available for steroid hormone synthesis.

CYP21 is responsible for aldosterone and corticosteroid synthesis, which are essential for stress response, blood pressure salt–water balance and other functions in the body. In the current study, TBC significantly repressed the forskolin-induced *CYP21* mRNA expression and decreased further with increasing TBC concentrations. Inhibition of *CYP21* expression may lead to decrease cortisol and aldosterone synthesis. However, the level of cortisol and aldosterone production was not measured in this experiment. A recent study reported that 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN), a kind of polycyclic musks used in personal care products inhibited expression of *CYP21* gene along with reduced production of cortisol (Li et al. 2013). If TBC inhibited cortisol and aldosterone synthesis, it could possibly disturb cortical hormone balance and may cause health problems related with glycometabolism (Løvås and Husebye 2007).

Fig. 3 Schematics of steroidogenesis processes. A summary of results of hormone production and gene expression after exposure of forskolin-stimulated H295R cells to 1 μ M TBC. Hormones analyzed are typed in **bold orange**, whereas gene expression analyzed is typed in *blue*. Upwards arrow up-regulation; downwards arrow down-regulation; asterisk statistical significance ($P < 0.05$)

3βHSD2 play a vital role in the adrenal biosynthesis of mineralocorticoids (aldosterone) and glucocorticoids (cortisol), but its expression is oppositely correlated with adrenal androgens synthesis such as dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and androstenedione (Rainey and Nakamura 2008). CYP17 is a dual-functional enzyme that catalyzes the synthesis of both cortisol and androgens. 3βHSD2 can convert steroid synthesis from mineralocorticoids to glucocorticoids or weak androgens, whereas inhibition of CYP17 would have the inverse effect (Song et al. 2008). In the present study, we observed that TBC inhibited basal and forskolin-induced 3βHSD2 expression; therefore, it would raise testosterone levels, but testosterone levels are lower, possibly due to down-regulation of CYP17 expression. The decrease in CYP17 gene expression could shift direction to the production of mineralocorticoids other than androgen synthesis. Interestingly, our results correlated well with a recent study that showed a significant reduction in the basal and agonist-induced 3βHSD2 and CYP17 gene expression along with inhibited androgen biosynthesis in the H295R cells after exposure to pioglitazone (Kempná et al. 2007).

CYP19 encodes aromatase, an important enzyme in the body for estrogen synthesis that oxidizes and aromatizes androstenedione and estrone and E2, respectively (Fig. 3). The inhibition production of E2 was likely related to reduce CYP19 gene expression. Reduction of CYP19 gene expression might result in a loss of aromatase enzyme

concentrations and activities. It has been hypothesized that the alteration of aromatase activity by various chemicals represents a potential mechanism of endocrine disruption (Cantón et al. 2005; Sanderson et al. 2000, 2004). It is possible that TBC might exert anti-estrogenic effects via aromatase inhibition if this mechanism could occur in vitro and in vivo models. Thus, further researches are required to validate this hypothesis.

The protein encoded by the *StAR* gene is one of the important enzymes in steroid hormone synthesis, which delivers cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where it is cleaved to pregnenolone by CYP11A, and this process is the rate-limiting step in steroidogenesis (Stocco 1999). Down-regulation of *StAR* expression would inhibit steroidogenesis by decreasing the availability of the precursor substance for the first conversion reaction occurring in the inner mitochondrial membrane. Our study founded that exposure of H295R cells to TBC did not affect the *StAR* mRNA expression, but when co-exposed with forskolin, *StAR* mRNA expression obviously decreased in the two highest concentration groups ($P < 0.5$). Alteration in forskolin-induced *StAR* mRNA expression implied that TBC might affect steroid synthesis and eventually lead to a decrease in T and E2. A study showed that the expression of *StAR* gene was suppressed by o,p'-DDD under basal and stimulation condition (Lin et al. 2012). Likewise, another study reported that transcript expression of *StAR* gene was

inhibited by some PCBs (Hilscherova et al. 2004). However, polybrominated biphenyls (PBBs 169) induced the *StAR* gene expression at low concentration (Ding et al. 2007). Although *StAR* gene expression was down- or up-regulated after exposure to chemicals, researchers demonstrated that the increase in *StAR* protein concentrations in monkey kidney cell line (COS-1) was not caused by an inducement in *StAR* gene expression other than an increase in stabilization of the *StAR* protein (Sugawara and Fujimoto 2004).

Conclusion

According to the results, it showed that TBC inhibited the synthesis of sex hormones (E2 and testosterone) in vitro because it extensively interfered with the mRNA level of adrenal steroidogenic enzymes including *StAR*, 3β HSD2, CYP17, CYP21 and CYP19 under basal and/or forskolin-stimulating condition in the H295R cells. The present study further supports the mechanism of action of chemical agent-induced endocrine-disruptive effects via alteration of steroidogenic genes, which could interfere with steroid hormone synthesis and, in turn, affect homeostasis and organism health. The H295R cell line serves as a useful in vitro model for adrenocortical toxicity researches and screening suspected endocrine disruptors. More work is required to establish whether TBC could impact on adrenocortical gland in vivo.

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