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Effect of tributyltin on mammalian endothelial cell integrity

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ABSTRACT

Tributyltin (TBT), is a man-made pollutants, known to accumulate along the food chain, acting as an endocrine disruptor in marine organisms, with toxic and adverse effects in many tissues including vascular system. Based on the absence of specific studies of TBT effects on endothelial cells, we aimed to evaluate the toxicity of TBT on primary culture of porcine aortic endothelial cells (pAECs), pig being an excellent model to study human cardiovascular disease. pAECs were exposed for 24 h to TBT (100, 250, 500, 750 and 1000 nM) showing a dose dependent decrease in cell viability through both apoptosis and necrosis. Moreover the ability of TBT (100 and 500 nM) to influence endothelial gene expression was investigated at 1, 7 and 15 h of treatment. Gene expression of tight junction molecules, occludin (OCLN) and tight junction protein-1 (ZO-1) was reduced while monocyte adhesion and adhesion molecules ICAM-1 and VCAM-1 (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) levels increased significantly at 1 h. IL-6 and estrogen receptors 1 and 2 (ESR-1 and ESR-2) mRNAs, after a transient decrease, reached the maximum levels after 15 h of exposure. Finally, we demonstrated that TBT altered endothelial functionality greatly increasing monocyte adhesion. These findings indicate that TBT deeply alters endothelial profile, disrupting their structure and interfering with their ability to interact with molecules and other cells.

1. Introduction

Among man-made pollutants, known to accumulate along the food chain, organotins, and mainly trisubstituted tin compounds, are especially dangerous, due to their wide industrial exploitation as polyvinyl chloride stabilizers, catalysts, pesticides and biocides in antifouling paints.

Tributyltin (TBT) interact by both covalent and non-covalent bonds with biomolecules and membrane structures and are considered among the most toxic substances ever deliberately introduced into environment (Pagliarani et al., 2013).

In spite of bans of TBT use (IMO 2001), the residue in marine environment is still an important concern (Horiguchi, 2012) due to its environmental persistence (Hoch, 2001; Fent, 2004). The contamination of aquatic environments is especially harmful. Bioaccumulation in tissues of exposed species (Frouin et al., 2010) leads to contamination of seafoods (Ma et al., 2011) and in turn, mainly through the food chain, of terrestrial species including human (Kannan et al., 1999; Takahashi et al., 1999).

TBT exerts a toxic effect acting as a classical endocrine disruptor for marine organisms causing imposex in gastropod mollusks (Gallo and Tosti, 2013). TBT shows also toxic and adverse effects in many kinds of cells and tissues of a variety of species, including mammalian (Ohshima et al., 2005) in which affects endocrine system through different pathway. Organotins are potent inhibitors of 11β-hydroxysteroid dehydrogenase type-2 (11β-HSD2) (Atanasov et al., 2005) and shows proadipogenic activity in some cell lineages like human and mouse multipotent stromal stem cells (Kirchner et al., 2010; Li et al., 2011; Penza et al., 2011).

Different cell types have depicted dissimilar levels of tolerance to TBT, resulting in a diversity of effects and in specific toxic concentrations for every cell lineage. In a cultured human granulosa-like tumor cell line, Saitoh et al. (2001) found a toxic TBT concentration of 1000 ng/mL, causing cell death within 24 h, while 200 ng/mL induced apoptosis of the cells. In neurons continuously exposed to TBT for 3 days, Yamada et al. (2010) observed a TBT-induced death at 30 nM in 4–6 days of culture and at 50 nM in 14–16 days of culture, which means that older neurons are more resistant to TBT toxicity. Significant loss of viability was observed in neuroblastoma cells incubated for 24 h with doses of TBT ranging from 250 nM onward, with a linearity found between 250 nM and 2 μM (Ferreira et al., 2013) and in a Sertoli-germ cell co-culture incubated for 6 h with a minimum dose of 300 nM (Mitra et al., 2013b).

Abbreviations: (pAECs), porcine aortic endothelial cells; (TBT), tributyltin; (OCLN), occludin; (ZO-1), tight junction protein-1; (ICAM-1), intercellular adhesion molecule-1; (VCAM-1), vascular cell adhesion molecule-1; (IL-6), interleukin-6; (ESR-1 and ESR-2), estrogen receptors 1 and 2.

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The information on human exposure to butyltin compounds is limited; some studies found TBT, DBT (dibutyltin), and MBT (monobutyltin) levels in human tissues in the range of 3–100 nM (Kannan et al., 1999; Takahashi et al., 1999). Butyltin compounds were already found in human blood in concentrations ranging between 64 and 155 ng/mL (Whalen et al., 1999), in particular TBT have been found up to 261 nM. This variability could be related to human diet, food habits, gender and physiological stage which should be taken into account; controlled trial in animal models could overcome this problem.

Recently, very interesting studies, using rodent model, correlated TBT to cardiovascular disorders impairing the coronary vascular reactivity response to estradiol and producing endothelial denudation in isolated rat heart (Dos Santos et al., 2012) and demonstrated TBT ability to reduce vasoconstrictor response in isolated aortic rings of female rats (Rodrigues et al., 2014).

Among cellular components of vasculature, the endothelium is especially susceptive to plasma toxicants because it is structurally arranged in a single layer of cells that first come into contact with blood vessel contents. Further, injuries to endothelial cells are implicated in the pathophysiology of several diseases (Yamada et al., 2011) and in particular in the cardiovascular ones (Mordi and Tzemos, 2014).

Based on the recent demonstration that TBT can influence vascular system and on the absence of specific studies of TBT effects on endothelial cells, we aimed to evaluate the toxicity of TBT on mammalian endothelial cells, using primary cultures of porcine aortic endothelial cells (pAECs), swine being an excellent animal model in the field of cardiovascular research (Forni et al., 2005; Vilahur et al., 2011; Zaragoza et al., 2011; Zannoni et al., 2012; Gessaroli et al., 2012). In addition, we aimed to investigate the ability of TBT to influence gene expression of markers involved in structure and function maintenance and in response to injuries of endothelial cells and in estrogen sensitivity. Finally we evaluate the alteration of endothelial function induced by TBT through monocyte adhesion assay.

2. Materials and methods

2.1. Chemicals and reagents

Human endothelial SFM medium, heat inactivated FBS (fetal bovine serum) and fungizone were purchased from Gibco-Life technologies. Trypsin-EDTA solution 1×, dimethyl sulfoxide (DMSO) and tributyltin chloride (TBT) were from Sigma-Aldrich and Dulbecco's phosphate buffered saline (DPBS) from EuroClone. AlexaFluor 488 Annexin/dead cell apoptosis kit (Molecular Probes, Eugene, USA Invitrogen) and CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega. Promega Corporation 2800 Woods Hollow Road Madison, WI 53711 USA) were used. NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG Postfach 10 13 52 D-52313 Düren Germany) was used for RNA isolation and IScript cDNA synthesis kit, IQ Supermix and IQ SyBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used for cDNA synthesis and RT-PCR analysis.

2.2. Cell culture

pAECs were isolated and maintained as previously described by Bernardini et al. (2005) and used from the third to the sixth passage. The first seeding after thawing was always performed in T-25 tissue culture flasks (3×10^5 cells/flask) (T25-Falcon, Beckton-Dickinson, Franklin Lakes, NJ, USA) and successive experiments were conducted in 24-well (qPCR analysis and monocyte adhesion assay) or 96-well assay plates (cell viability) (Falcon Beckton-Dickinson) with confluent cultures. Cells were cultured in human endothelial SFM medium, added with FBS (5%) and antimicrobial/antimycotic solution (1× Gibco-Life technologies code 15240-062) at 38.5 °C. The tributyltin chloride was diluted in DMSO until a 5 mM solution and therefore in culture medium to obtain desired concentrations for cell exposure.

2.3. Cell viability

The ability of TBT to induce cytotoxicity was evaluated by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega BioSciences LLC San Luis Obispo, CA, USA) that quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis.

Confluent pAECs were incubated for 24 h in a 96 well plate with TBT (0, 100, 250, 500, 750, and 1000 nM) and the supernatant was collected to be analyzed. The maximum LDH activity was assessed by lysing cells, collecting the resulting medium and processing it as other samples. Briefly, supernatant was incubated with a reconstructed substrate mix for 30 min at room temperature, protected from light. Then, a stop solution was added and the absorbance was recorded at 490 nm. Cytotoxicity of TBT was calculated according to the equation:% Cytotoxicity = Experimental LDH release (OD 490) / Maximum LDH release (OD 490).

To determine the ability of TBT to induce apoptosis or necrosis the Annexin V binding assay was employed. This assay detects phosphatidilserine (PS) externalization on the plasma membrane.

In viable cells PS is located on the cytoplasmic surface of the cell membrane. However during the early stage of apoptosis, PS is translocated from the inner to the outer leaflet of the plasma membrane.

Confluent pAECs cultures were incubated in a 24 well plate with increasing doses of TBT and the Annexin V/PI binding assay (Alexa Fluor® 488, Life Technologies) was used.

Cells were harvested, placed in Eppendorf tubes, centrifuged at $500 \times g$ for 10 min and resuspended in 100 μL of Annexin binding buffer. Annexin V-FITC (5 μL) and propidium iodide (1 μL) were added to cell suspension. After incubation, the cells were analyzed with a flow cytometer (FACSAria; BD Biosciences) by collecting at least 10^4 events.

2.4. RNA isolation and quantitative real time PCR (qPCR)

To determine the ability of TBT to influence endothelial gene expression, confluent pAECs were incubated for different times (1, 7, and 15 h) with different doses of TBT (0, 100 or 500 nM).

Total RNA was isolated using the NucleoSpin®RNA Kit (Macherey-Nagel GmbH & Co. KG, Germany), and 1 μg of total high quality RNA (A_{260}/A_{280} ratio above 2.0) was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA) in a final volume of 20 μL. Swine primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA) for each studied gene: estrogen receptor 1 and 2 (ER-1; ER-2); tight junction proteins: occludin (OCLN) and tight junction protein-1 (ZO-1); adhesion molecules: vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1); cytokine: interleukin-6 (IL-6). Moreover, primers for the following reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and hypoxanthine-guanine phosphoribosyltransferase (HPRT-) were designed in order to evaluate the most suitable using BestKeeper Software (Pfaffl et al., 2004) for qPCR normalization. Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Table 1. Quantitative real-time PCR was performed to evaluate gene expression profiles in iCycler (Bio-RAD) using SYBR green I detection system. The amplification reaction (25 μL) contained 12.5 μL of IQ SYBER Green Bio-RAD Supermix (Bio-RAD), 1 μL of each forward and reverse primer (5 μM), 2.5 μL cDNA and 8 μL of water. All samples were performed in duplicate and controls lacking cDNA template were included to determine the specificity of target amplification. The real-time program included an initial denaturation for 1 min 30s at 95 °C, 40 cycles of 95 °C for 15 s, and 60 °C for 30s, followed by a melting step with ramping from 55 °C to 95 °C at a rate of 0.5 °C/10s. Specificity of the amplified PCR products was confirmed by melting curve analyses and agarose gel electrophoresis. The expression level of interest genes was calculated as fold of increase using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) in relation to pAECs cultured under standard conditions (control).