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Tributyltin contributes in reducing the vascular reactivity to phenylephrine in isolated aortic rings from female rats

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HIGHLIGHTS

- Tributyltin (TBT) reduces vasoconstrictor response in aortic rings of female rats.
- TBT decreases smooth muscle actin protein expression in aortic rings of female rats.
- TBT induces an endothelial dysfunction in aortic rings of female rats.
- Collagen deposition along aortic wall after 15 days of TBT exposure in female rats.
- TBT changes vascular reactivity dependent on NO, K^+ channels and oxidative stress.

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ABSTRACT

Organotin compounds such as tributyltin (TBT) are used as antifouling paints by shipping companies. TBT inhibits the aromatase responsible for the transformation of testosterone into estrogen. Our hypothesis is that TBT modulates the vascular reactivity of female rats. Female Wistar rats were treated daily (Control; CONT) or TBT (100 ng/kg) for 15 days. Rings from thoracic aortas were incubated with phenylephrine (PHE, 10^{-10}–10^{-4} M) in the presence and absence of endothelium, and in the presence of N^G-Nitro-L-Arginine Methyl Ester (L-NAME), tetraethylammonium (TEA) and apocynin. TBT decreased plasma levels of estrogen and the vascular response to PHE. In the TBT group, the vascular reactivity was increased in the absence of endothelium, L-NAME and TEA. The decrease in PHE reactivity during incubation with apocynin was more evident in the TBT group. The sensitivity to acetylcholine (ACh) and sodium nitroprusside (SNP) was reduced in the TBT group. TBT increased collagen, reduced α1-smooth muscle actin. Female rats treated with TBT for 15 days showed morphology alteration of the aorta and decreased their vascular reactivity, probably due to mechanisms dependent on nitric oxide (NO) bioavailability, K^+ channels and an increase in oxidative stress.

1. Introduction

Organotin compounds (OTCs) such as tributyltin (TBT) and triphenyltin (TPT) have been widely used as biocides, agricultural fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters, as well as in antifouling paints for marine vessels (Piver, 1973). The toxicity level of OTCs may be related to the concentration, exposure time, bioavailability, and sensitivity of the biota, as well as the persistence of OTCs in the environment (de Carvalho Oliveira and Santelli, 2010).

An increase in the toxicity of organotin compounds can be related to their insolubility in water, and their high lipophilicity is the main parameter leading to bioaccumulation in food webs

Abbreviations: L-NAME, N^G-Nitro-L-Arginine Methyl Ester; TBT, tributyltin; PHE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; CONT, Control; NO, nitric oxide; TEA, tetraethylammonium; SR, sarcoplasmic reticulum; OTCs, Organotin compounds; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells; COX-2, cyclooxygenase -2.

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(Fent, 2004). OTCs, especially triorganotins, induce an endocrine syndrome in some species of gastropods, known as imposex. This syndrome is characterized by the superimposition of male genitalia in females, which alters their reproductive function (Smith, 1981). The mechanism of action remains unclear, but appears to be related to the direct inhibition of the aromatase enzyme of cytochrome P_450 that converts testosterone to estradiol (Snyder et al., 1999). In female rats, TBT has been shown to modulate estradiol, alters the estrous cycle, impedes the development of ovarian follicles, induces an imbalance of ovarian hormones and reduces the number of gonocytes and germ cells, thereby affecting sexual development (Grote et al., 2004). Its effects on other functions are not well understood. Furthermore, TPT decreases aromatase activity in ovary cells (Nakanishi et al., 2002).

Estrogen is a steroid hormone involved mainly in the control of female reproductive functions. Among other effects, estrogen can be cardioprotective, causing endothelium-dependent and-independent vasodilation of coronary arteries isolated (Crews and Khalil, 1999). Estrogen treatment also increases aortic stiffness and potentiates endothelial vasodilator function in the hindquarters (Tostes et al., 2003).

According to in vitro results, both the sarcoplasmic reticulum (SR) Ca^{2+} ATPase and Ca^{2+} uptake were inhibited significantly in rats treated with these stannous compounds, indicating a transport inhibition of cardiac SR Ca^{2+} (Kodavanti et al., 1991). Based on the knowledge that estradiol can modulate the cardiovascular system through both endothelium-dependent and independent vasodilation, our hypothesis was that TBT can increase the vascular reactivity of isolated aortic rings of female rats.

2. Materials and methods

2.1. Chemicals

The following materials were used for the experiments: L- phenylephrine hydrochloride (PHE), ACh chloride (ACh), N^G-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME), tetraethylammonium (TEA) (Sigma Chemical Co., St. Louis, Missouri, USA), tributyltin chloride (TBT, 96% Sigma, St. Louis, Mo., USA), sodium phenobarbital (Fontoveter, Brazil), and sodium nitroprusside (SNP) (Merck & Co., Inc., Rahway, NY, USA). These chemicals were dissolved in distilled water, except TBT, which was dissolved in 0.4% ethanol. Salts and reagents used were of analytical grade.

2.2. Experimental animals

Female Wistar rats (12 weeks old), were kept under controlled temperature between 23–25 °C with 12:12 h light/dark cycle. Rat chow and filtered tap water were provided *at libitum*. All protocols were approved by the Local Ethics Committee of Animals (CEUA-UFES 020/2009). The rats were divided into two groups: Control (CONT, $n = 8$) were treated daily with vehicle (ethanol 0.4%), and TBT-treated rats were treated with TBT (TBT, 100 ng kg^{-1} day^{-1} diluted in 0.4% ethanol vehicle, $n = 8$) by gavage for 15 days. All the animals were anesthetized with sodium phenobarbital (35 mg/kg, ip) before the surgical procedures.

2.3. Hormonal measurement

Immediately prior to removing the thoracic aorta, blood samples were collected via puncture of the abdominal aorta to measure the circulating levels of sex hormones. The blood samples were immediately centrifuged at 825 × *g* at 4 °C for 10 min to obtain plasma, which was stored at −20 °C for future measurements of progesterone and estradiol by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA).

2.4. Tissue preparation

Animals were perfused with sterile saline containing heparin (10 U/mL) via the left cardiac ventricle followed by infusion with PBS-formalin. The aorta was removed and manually dissected into rings (3–4 mm, $n = 5$ per group), fixed in PBS-formalin, pH 7.4, for 24–48 h at room temperature. After fixation, tissues were dehydrated in graded ethanol, cleared in xylol, and embedded in paraffin at 60 °C and further sectioned into 5 μm slices. Sections were stained with H&E to visualize the aortic vascular tunics (Barreira et al., 2009). Processing and microscopic analysis were performed at the LUCCAR Lab, Federal University of Espírito Santo, Brazil.

2.5. Histomorphometry

Histomorphometry image analysis system was composed of a digital camera (Axio-Cam ERc 5S) coupled to a light microscope (Olympus AX70; Olympus, Center Valley, PA). High resolution images (2048 × 1536 pixels buffer) were captured with Carl Zeiss AxioVision Rel. 4.8. Photomicrographs were obtained using a 10× objective, and the thickness of aortic wall (which included all vascular tunicas/field) and the aortic wall area were calculated with the area measure tool of AxioVision Rel. 4.8. The results represent the thickness and the area of aortic wall and are expressed as the mean ±SEM.

2.6. Collagen density surfaces

Mallory trichrome stained sections were used to obtain 15 photomicrographs from aortic tissue with a 20× objective lens. The areas were randomly chosen, and areas without all vascular tunicas were carefully avoided. The random fields from each well are photographed under phase contrast and analyzed in Image J. The images were converted into high-contrast back and white images to visualize collagen fibers stained. The results represent the percentage of collagen in the total aortic wall surface and it is expressed as the mean ±SEM, as described by dos Santos et al. (2012).

2.7. Protein extraction and immunoblotting

The animals were sacrificed and the aortas were removed and manually dissected into rings (3–4 mm, $n = 5$ per group) as described previously by Fiorim et al. (2011). The tissues were homogenized in lysis buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 20 mmol/L imidazole, pH 7.2, and the following protease inhibitors: 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mmol/L benzamide, 10 mg/L leupeptin, 1 mg/L pepstatin A, 1 mg/L aprotinin, and 1 mg/L chymostatin). Homogenization was carried out at 0 °C using a Potter homogenizer. The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was saved, the pellet was suspended in three volumes of lysis buffer, and the centrifugation was repeated. Both supernatants were mixed and centrifuged at 10,000 × *g* for 20 min. The supernatant was saved and the pellet was discarded. The protein concentration was determined using the Lowry assay (1951). Proteins were solubilized by heating at 100 °C for 1 min in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, 0.01% bromophenol blue, and 1.7% β-mercaptoethanol). Standard SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by loading equal quantities of protein per lane (20 μg) on to 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) in Tris–glycine transfer buffer. The membranes were blocked with 5% (bovine serum) albumin (BSA, Sigma–Aldrich) in a Tris-buffered saline 0.05% Tween 20 solution (TBS-T) for 1 h at 4 °C, washed once for 10 min in TBS-T and then incubated with a mouse monoclonal antibody raised against the C-terminal domain of α1-smooth muscle actin (α-SMA, diluted 1:500 in 3% BSA in TBS-T overnight at 4 °C) (Santa Cruz Biotechnology, INC) or a rabbit polyclonal antibody raised against a peptide mapping region near the C-terminus of rat β-Actin (β-Actin, diluted 1:1000 in 3% BSA in TBS-T overnight at 4 °C) (Santa Cruz Biotechnology, INC). After incubation with the primary antibody, the membranes were washed three times with TBS-T for 10 min each wash. The α-SMA protein was detected using a secondary anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:1000 in 3% BSA in TBS-T for 1 h at 4 °C) (Sigma Immuno-Chemicals), whereas the β-actin antibody was detected using an anti-mouse IgG alkaline phosphatase conjugate (diluted 1:4000 in 3% BSA in TBS-T for 1 h at 4 °C) (from Sigma Immuno Chemicals). The blots for α-SMA and their respective β-actin (used as internal control) were visualized by a color development reaction using nitroblue tetrazolium chloride (NBT) and 50 mg/mL of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (all from Life Technologies, Rockville, MD) for 5 min. The α-SMA and β-actin bands were analyzed by densitometry using Image J software. Relative expression was normalized by dividing the α-SMA value by the corresponding internal control values (β-actin).

2.8. Superoxide anion detection

To detect O_2^- level in the thoracic aorta, cryosections (8 μm) of aorta in Tissue-Tek optimized cutting temperature (OCT) were allowed to thaw and incubated with O_2^- sensitive fluorescent dyedihydroethidium (DHE) (2 μM) at 37 °C for 30 min in the dark. The images were obtained by confocal fluorescent microscope (Leica DM 2500). Fluorescence was detected at 585 nm. The signal intensity within the media layer was analyzed in the whole circumference of 3 sections the vessel by a blinded researcher.

2.9. Tissue bath studies

All the animals were anesthetized with sodium phenobarbital (35 mg/kg, ip) and killed by exsanguination. A section of the thoracic aorta was removed and placed in oxygenated Krebs-Henseilet bicarbonate buffer of the following composition: 131 mM NaCl, 4.7 mM KCl, 18 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, and 0.01 mM EDTA. The buffer was kept at 36.5 °C and was gassed with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. The aorta was cleaned