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Exposure of Jurkat cells to bis (tri-n-butyltin) oxide (TBTO) induces transcriptomics changes indicative for ER- and oxidative stress, T cell activation and apoptosis

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ABSTRACT

Tributyltin oxide (TBTO) is an organotin compound that is widely used as a biocide in agriculture and as an antifouling agent in paints. TBTO is toxic for many cell types, particularly immune cells. The present study aimed to identify the effects of TBTO on the human T lymphocyte cell line Jurkat. Cells were treated with 0.2 and 0.5 μM TBTO for 3, 6, 12 and 24 h and then subjected to whole genome gene expression microarray analysis. The biological interpretation of the gene expression profiles revealed that endoplasmic reticulum (ER) stress is among the earliest effects of TBTO. Simultaneously or shortly thereafter, oxidative stress, activation of NFKB and NFAT, T cell activation, and apoptosis are induced. The effects of TBTO on genes involved in ER stress, NFAT pathway, T cell activation and apoptosis were confirmed by qRT-PCR. Activation and nuclear translocation of NFATC1 and the oxidative stress response proteins NRF2 and KEAP1 were confirmed by immunocytology. Taking advantage of previously published microarray data, we demonstrated that the induction of ER stress, oxidative stress, T cell activation and apoptosis by TBTO is not unique for Jurkat cells but does also occur in mouse thymocytes both *ex vivo* and *in vivo* and rat thymocytes *ex vivo*. We propose that the induction of ER stress leading to a T cell activation response is a major factor in the higher sensitivity of immune cells above other types of cells for TBTO.

Introduction

Tributyltin-oxide (TBTO; $C_{24}H_{54}OSn_2$) belongs to the organotin family and is widely used as a broad spectrum biocide in agriculture and various industries. TBTO is also used as a catalyst in polyurethane, as a stabilizer in plastic industry and as disinfectant in paper mills (Snoeij et al., 1987; Fent, 1996; Antizar-Ladislao, 2008). Due to its strong biocidal activity TBTO is used as antifouling agent in paints for conservation of timber products (Granmo et al., 2002; Camps et al., 2011). As a consequence of its extensive usage, TBTO is widely spread and accumulated in the environment (Hoch, 2001). Humans are exposed to TBTO primarily through the consumption of contaminated meat and fish products or via leakage from plastic products (Boyer, 1989; Kannan et al., 1995; Iwata et al., 1997; Takahashi et al., 1999).

Previous studies have indicated that TBTO is immunotoxic, neurotoxic and hepatotoxic (Vos et al., 1984; Snoeij et al., 1987; O'Callaghan and Miller, 1988; Yoshizuka et al., 1992; Tsunoda et al., 2004; Baken et al., 2006). The most sensitive and critical toxicological end point of TBTO is immunotoxicity (Verdier et al., 1991; Penninks, 1993). TBTO induces immunosuppression in mice and rats by acting on cortical thymocytes and by repressing T cell mediated immune responses (Ishaaya et al., 1976; Vos et al., 1984; Vos et al., 1990). In vitro studies on human immune cells indicate that TBTO reduces NK cell, eosinophil and macrophage activity and thereby suppresses the immune response (Aluoch et al., 2006; Aluoch et al., 2007; Sroka et al., 2008). *In vivo* studies on rats showed that tributyltin induces DNA damage in blood cells and increased levels of reactive oxygen species in the liver (Liu et al., 2006). TBTO also inhibits DNA synthesis in thymocytes (Seinen et al., 1979; Snoeij et al., 1986). Furthermore, TBTO inhibits cell proliferation and induces apoptosis in rat thymocytes and human primary lymphocytes (Grundler et al., 2001; Berg et al., 2003; Baken et al., 2007). Proteomic analysis of the mouse thymoma cell line EL4 exposed to TBTO showed effects on the expression of proteins involved in cell structure and cytoskeleton, protein synthesis, protein trafficking and cell proliferation (Osman et al., 2009). In spite of these studies, the precise mechanism behind the action of TBTO has not yet been elucidated.

In the present study, we applied a toxicogenomic approach to examine the effects of TBTO on gene expression in the human T lymphocyte cell line Jurkat using whole genome microarray analysis. Jurkat cells were exposed to different doses of TBTO for 3, 6, 12 and

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24 h. TBTO treatment resulted in a rapid induction of gene expression changes. The biological interpretation of the microarray data led to the hypothesis that TBTO induces apoptosis via ER stress, rise of intracellular Ca^{2+} levels, NFAT and NFKB activation, T cell activation and oxidative stress. Analysis of previously published microarray data revealed that the processes affected by TBTO in Jurkat cells are also affected by TBTO *in vivo* in the mouse thymus as well as *ex vivo* in mouse and rat thymocytes.

Materials and methods

Chemicals. Bis-(tri-n-tributyltin) oxide (TBTO) and BAPTA were obtained from Sigma Aldrich Company (Zwijndrecht, The Netherlands). TBTO was 96% pure and was dissolved in 96% ethanol. Primary antibodies against NFATC1 (sc-17834/mouse monoclonal), NRF2 (sc-13032/rabbit polyclonal) and KEAP1 (sc-33569/rabbit polyclonal) were purchased from Santa Cruz Biotech (Santa Cruz, Heerhugowaard, The Netherlands). Secondary antibodies were goat anti mouse-IgG1-FITC/sc-2078 (Santa Cruz Biotech) and goat anti rabbit-IgG (H + L) FITC/28176-FITC-H488 (Anaspec, Heerhugowaard, The Netherlands).

Cell culture. The human T-lymphocyte cell line (Jurkat) was obtained from the American Type Culture Collection (ATCC). The Jurkat cells were grown in culture flasks in RPMI-1640 medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere. The medium was refreshed every 2 days. The endotoxin levels in the serum were <10 EU/ml.

WST-1 assay. Cell viability was selected as a parameter for determining the appropriate conditions for our microarray experiments. We measured cell viability using the WST-1 assay (Roche Diagnostics GmbH, Germany). WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) is a water soluble tetrazonium salt which is cleaved by mitochondrial dehydrogenases to form a colored formazan complex and the amount of formazan correlates to the viability of the cells. Cells (25,000 per well) were exposed in triplicate in 100 μl medium in 96 well plates for various time periods to increasing concentrations of TBTO and to the vehicle control ethanol. During the last 2 h of exposure, 10 μl of WST-1 reagent was added. Absorbance was measured at 450 nm in a microplate reader (BioTek, Winooski Vermont, USA).

Exposures. TBTO stock solution was diluted in RPMI-1640 medium without FCS. Jurkat cells at passage number 20 were used for the exposure experiments. On the basis of the results of the cell viability experiments, Jurkat cells were exposed in a volume of 3 ml in 6 well plates to 0.2 and 0.5 μM of TBTO for 3, 6, 12 and 24 h, thus making a total of 8 treatment groups. 750,000 cells were pipetted in 2.7 ml medium per well in 6-well plates. After growing the cells for 20 h, exposure was initiated by adding 0.3 ml medium with 10-times stock solution of TBTO or ethanol as vehicle control. The final ethanol concentration in the medium was kept equal for all treatment groups and was less than 0.1% which is not exerting any effect on cell viability. Exposures were performed in triplicate. To test the role of calcium in the toxicity of TBTO, Jurkat cells were exposed to TBTO for 3 and 6 h in the presence or absence of Ca^{2+} chelator BAPTA (4 μM) and cell viability was measured with the WST-1 assay.

RNA isolation. After exposure, cells were collected by centrifugation and washed with phosphate buffered saline (PBS). The cell pellet was solubilized in 600 μl red cell lysis buffer (RLT) (Qiagen, Venlo, The Netherlands) supplemented with 10% β-mercaptoethanol and stored at −80 °C. RNA isolation was performed with the Qiagen RNAeasy kit following manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Wilmington, USA) by measuring absorption ratios at 260/280 and 230/280 nm. The integrity of the RNA samples was examined using Bio-Rad's Experion system (Veenendaal, The Netherlands).

Microarray hybridizations. RNA samples were amplified and converted into Cy5-labeled cRNA using the Agilent low RNA input fluorescent amplification kit according to the manufacturer's instructions (Agilent Technologies, Amstelveen, The Netherlands). Universal human reference RNA (Stratagene, La Jolla, CA) was used as common reference and was labeled with fluorescent Cy3 dye (Perkin-Elmer/NEN). Equal amounts of Cy5-labeled cRNA and Cy3-labeled reference cRNA were mixed. Hybridizations were carried out on the 4×44K human whole genome Agilent microarray platform following the Agilent two-color microarray-based gene expression analysis protocol. Microarray slides were incubated for 16 to 17 h at 65 °C in a microarray incubation chamber with continuous rotation. After hybridization, the arrays were washed and dried at RT according to the manufacturer's protocol. Arrays were scanned using an Agilent microarray scanner (G2565B). In total, 36 arrays were hybridized.

Feature extraction 9.1 software (Agilent Technologies) was used for quantification of spot intensities. Quality check of arrays was carried out in R with LimmaGUI. GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium) was used for background correction and normalization (Pellis et al., 2003). Further possible errors such as efficiency and quality of probe labeling between various experiments were corrected (Pellis et al., 2003). To obtain an impression of the number of genes affected per treatment, genes were selected on the basis of >1.5 fold up- or down-regulation in combination with a p-value <0.01 according to Student's *T*-test.

Hierarchical clustering was performed with the programs Cluster (uncentered correlation; average linkage clustering) and Treeview (Eisen et al., 1998).

Biological interpretation

Metacore analysis. For genes within the subclusters of the hierarchical cluster heatmap, pathway analysis was done using Metacore software. This is an online program for functional and biological interpretation of gene expression data. Metacore uses hypergeometric distribution to assess significances for overrepresentation of affected genes in signaling and metabolic pathways (Ekins et al., 2006).

Gene set enrichment analysis (GSEA). GSEA is a statistical analysis tool for microarray data, used to detect biological processes affected and to provide insight into the affected molecular mechanisms. GSEA makes use of predefined gene sets that are based on previous experimental results and literature. GSEA ranks all the genes on their expression ratios and determines whether a particular gene set is significantly enriched at the top or the bottom of the ranked list (Subramanian et al., 2005). We used gene sets from various sources, including the cell cycle database based on results published by Whitfield et al.(2002) and Bar-Joseph et al.(2008), the lymphocyte database (Shaffer et al., 2001), Tox-action (self-made), Tox-TF target database (self-made), Biocarta-2 (http://www.biocarta.com/) and Gene Ontology (http://www.geneontology.org/). Significantly up and downregulated genes were selected for making heat maps of major biological processes and pathways.

Molecular concept analysis. Molecular concept analysis enables to visualize networks in which the overlap between gene sets based on co-occurrence of genes are shown (Rhodes et al., 2007). The overlaps between various gene sets were calculated based on the genes that were responsible for a gene set to be significantly affected. For this, either the top-20% or the bottom-20% of the genes were used for upregulated or downregulated gene sets respectively. Significant gene