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Triphenyltin enhances the neutrophilic differentiation of promyelocytic HL-60 cells☆

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Abstract

Triphenyltin (TPT) is an environmental endocrine disruptor and toxic substance, but little information is available on its immunological effects. To assess the effect of TPT on leukocyte differentiation, we investigated its effect on the neutrophilic differentiation of HL-60 cells induced by dimethyl sulfoxide and granulocyte colony-stimulating factor (G-CSF) for 6 days. At a low concentration, 10^{-7} M, TPT increased superoxide production by differentiated HL-60 cells stimulated with opsonized zymosan (OZ) by about 45% and increased expression of CD18, a component of the OZ-receptor, by about 90%. Real-time PCR analysis revealed that TPT augmented the expression not only of CD18 but also of components of superoxide-generating NADPH-oxidase, p47phox, 2.7-fold, and p67phox, 2.0-fold, and of granulocyte colony-stimulating factor receptor (G-CSFR), 3.0-fold, whereas various other endocrine disruptors, including parathion, vinclozolin, and bisphenol A, had no such enhancing effects. The results of a DNA macroarray analysis showed that TPT enhanced the expression of G-CSFR and certain other neutrophil functional proteins, including CD14 and myeloid leukemia cell differentiation protein (MCL-1), and that TPT induced a decrease in expression of LC-PTP, leukocyte protein–tyrosine phosphatase, to about half the control level. The TPT-dependent suppression of LC-PTP was confirmed by real-time PCR analysis, and the results of immunoblotting indicated that TPT enhances the expression of myeloid specific tyrosine kinase hck by about 30% at the protein level, and this together with the reduction of LC-PTP may enhance tyrosine phosphorylation, in turn resulting in enhancement of superoxide production. These findings suggest that TPT may have an enhancing effect on the neutrophilic maturation of leukocytes.

Keywords: Triphenyltin; Neutrophil; Differentiation; Respiratory burst; Superoxide; CD18; Hck; Opsonized zymosan; HL-60 cell

Triphenyltin (TPT) is an organotin compound that has been widely used as an anti-fouling agent in paints applied to ships, aquaculture nets, and docks, and as a slimecide for cooling towers and wood prevention. Environmental organotin compounds have recently been shown to induce a masculinization process involving the development of male sex organs in female mollusks, called ''imposex'' [1]. It is a clear example of the endocrine-disrupting effect of environmental chemicals on wild animals and serious public attention is paid to organotin compounds [2]. On the other hand, there are several reports describing that TPT has immunosuppressive [3–5] and apoptosis-inducing effects [6].

Recent studies have revealed that the immune, nervous, and endocrine systems are interlinked. Many of the same cytokines and their receptors are expressed by all three systems, and steroid hormones, including estrogen, testosterone, and glucocorticoids, have regulatory functions that affect immunocompetent cells [7]. Endocrine disruptors are generally thought to be capable of affecting the development or maturation of reproductive organs at low doses during ontogeny [2]. Thus, it seemed appropriate to investigate the effect of such chemicals on the maturation process of immuno-

☆ *Abbreviations:* TPT, triphenyltin; OZ, opsonized zymosan; HBSS, Hanks' balanced salt solution; G-CSF, granulocyte colony-stimulating factor; LC-PTP, leukocyte protein–tyrosine phosphatase.

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competent cells in a sensitive experimental system in which low-dose effects could be detected reproducibly.

In this paper we describe the effect of TPT on the differentiation of HL-60 cells. This is the first report to show that TPT enhances the superoxide production triggered by opsonized zymosan (OZ) in clear correlation with the expression of CD18 (OZ-receptor). We also report that TPT is a unique endocrine disruptor that has a potent enhancing effect on the expression of p47phox and p67phox, the components of superoxide-generating NADPH oxidase [8].

Materials and methods

Cells. HL-60 cells obtained from the Japanese Cancer Research Resources Bank were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were induced to differentiate into neutrophil-like cells by exposure to 1.25% dimethyl sulfoxide (DMSO) and 25 ng/ml granulocyte colony-stimulating factor (G-CSF) for 6 days [9]. TPT was present during differentiation, and the terminally differentiated HL-60 cells were washed with HBSS and their characteristics were analyzed as described below.

Reagents. G-CSF was obtained from Chugai Pharmaceutical (Tokyo, Japan). OZ was prepared as described previously [10]. TPT was purchased from Wako Chemical (Osaka, Japan). All other chemicals used were commercially available products of the highest purity.

Antibodies. Monoclonal anti-CD18 antibody was obtained from Sigma-Genosys (The Woodlands, TX). Mouse IgG was from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody was purchased from Immunotech (Marseille, France). Rabbit anti-hck and goat anti-p22phox antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA), and anti-rabbit p47phox antibody was prepared as described previously [10]. Anti-cofilin monoclonal antibody was generously donated by Dr. T. Obinata and Dr. H. Abe (Chiba University, Chiba, Japan). Horseradish peroxidase-labeled anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Amersham Biosciences K.K. (Piscataway, NJ). Horseradish peroxidase-labeled anti-goat IgG antibody ($F(ab')_2$) was purchased from EY Laboratories (San Mateo, CA).

Determination of superoxide production and CD18 expression. The HL-60 cells differentiated in the presence of TPT were washed with HBSS and their OZ-stimulated O_2^- production was assayed using cytochrome *c* [10] or a chemiluminescence reagent [11] as described previously. Other differentiated cells (2×10^6) were washed with PBS, incubated with an anti-CD18 monoclonal antibody (final concentration 4 μg/ml) or mouse IgG (4 μg/ml) at 4 °C for 20 min, and then stained with the FITC-labeled anti-mouse IgG (15 μg/ml) at 4 °C for 20 min. The cells were washed with PBS and the fluorescence intensity of aliquots (10^4 cells) was analyzed with a flow cytometer (FACScan, BD, San Jose, CA).

Analysis of changes in gene expression of functional proteins. The changes in gene expression were investigated in RNA derived from the differentiated cells (day 4) by using a DNA membrane array (Atlas Human Hematology/Immunology Array, Clontech, Palo Alto, CA) and real-time PCR (PRISM 7000, Applied Biosystems (Foster City, CA)) according to the technical brochures of the companies. The primers and TaqMan probes used were: CD18, ACCAGCCCA GAGGTGACTGT (forward), CTGCTCCTGGATGCACTCTGT (reverse), and AATGTCCCGATCACCTTCCAGGTGAA (TaqMan probe); p47phox, CCCTGCTGGGCTTTGAGAA, CCGACAGGTC CTGCCATTT, and CGCTTCGTACCCAGCCAGCACTATGT; p67phox, CATTGGCTGCATGTACACTATCC, TGGAAGTAAGC CACTGCCAAGT, and AGGCCTTTACCAGAAGCATTAACCG AGACA; granulocyte colony-stimulating factor receptor (G-CSFR), GCAG CAGCGTCTGTCTGATG, GGCTGTTGCCCCAGTTCA, and CC TGCCCCACCTCAACCACACTCA; and leukocyte protein–tyrosine phosphatase (LC-PTP), CCAGAGCACAGCCGTTGA, GCAGTCGCACATGCTTCTTG, and CATGACCCAGCCTCCGC CTGAA.

Immunoblotting. The electrophoresis and immunoblotting conditions were described in detail previously [10,12]. Briefly, cell lysates derived from chemical-treated cells (1×10^4) were subjected to sodium dodecyl sulfate electrophoresis using 12.5% acrylamide gel, followed by immunoblotting with anti-hck, anti-p47phox, anti-p22phox, and anti-cofilin antibodies. The staining intensity of each band was measured with image analyzers (Lumino-CCD, ATTO, Tokyo, Japan and LAS 3000, Fuji Film, Tokyo, Japan).

Results

Effect of TPT on superoxide production by differentiated HL-60 cells

To investigate the effect of TPT on leukocyte differentiation, HL-60 cells, an undifferentiated promyelocytic cell line, were induced to differentiate into neutrophil-like cells with DMSO and G-CSF in the presence of TPT in a wide range of concentrations. After washing the differentiated cells with HBSS, changes in their ability to produce superoxide, one of the most important functions of neutrophils, were determined quantitatively. OZ, a serum-treated insoluble polysaccharide derived from yeast, was used as a stimulant, because it can be used as a physiological activator in vitro. As shown in Fig. 1, TPT increased OZ-triggered superoxide production at low concentrations (0.01–0.1 μM). Almost the same dose-dependent enhancement of superoxide production was observed when tributyltin was used instead of TPT (data not shown). These results were unexpected and seemed important because TPT had been considered to be an immunosuppressor at higher concentrations (micromolar range) [3–5]. At concentrations above 1 μM, TPT was toxic to the HL-60 cells in this experimental system, probably by inducing apoptosis [6].

Effect of TPT on CD18 expression

CD18 is a typical granulocyte differentiation marker and a subunit of the OZ-receptor referred to as Mac-1 [13]. The effect of TPT on CD18 expression was investigated by flow cytometry, and as shown in Fig. 2, TPT augmented the expression of CD18 about 2-fold at 0.1 μM. TPT appears to enhance OZ-dependent O_2^- production and expression of OZ-receptor in a similar dose-dependent manner.

TPT-induced changes in gene expression

We then analyzed the TPT-induced changes in gene expression by quantitative real-time PCR in comparison with various other endocrine disruptors. As shown in

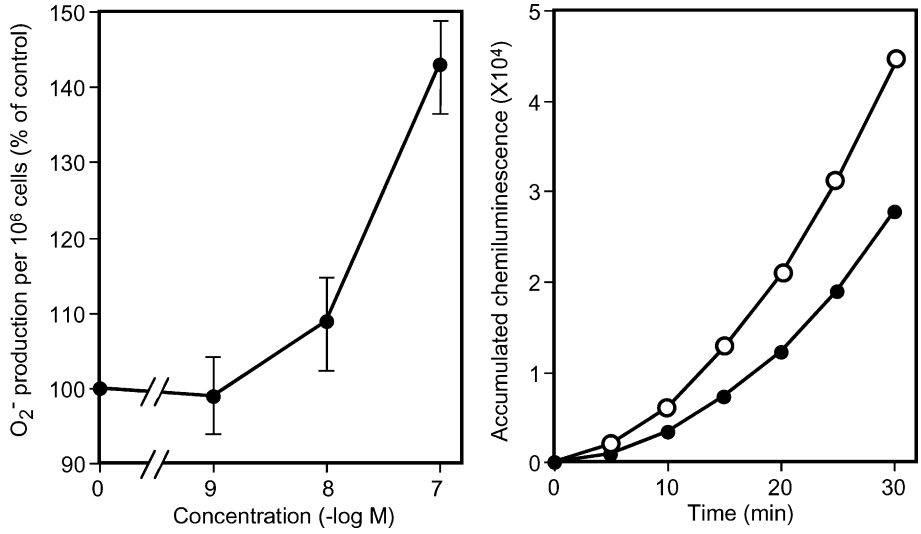


Fig. 1. TPT-enhanced superoxide production by neurophilic HL-60 cells. *Left*: HL-60 cells were induced to differentiate into neutrophil-like cells with DMSO and G-CSFR in the presence of TPT at the concentrations indicated. After 6-day culture for neutrophilic cell induction, the cells were washed with HBSS, and OZ-stimulated superoxide production was measured as a reduction of cytochrome *c* in 10 min. Three independent experiments were performed, and the amount of superoxide produced by 10^6 cells without TPT was normalized to 100%. The means and SDs are shown. The actual amounts of superoxide in the absence of TPT were 16–20 nmol/10 min/10^6 cells. *Right*: Time-course of superoxide production monitored by the chemiluminescence of 10^4 cells. The cells were differentiated as in the left panel in the absence (•) and presence (○) of 0.1 μM TPT. The details of the experiments are described in Materials and methods.

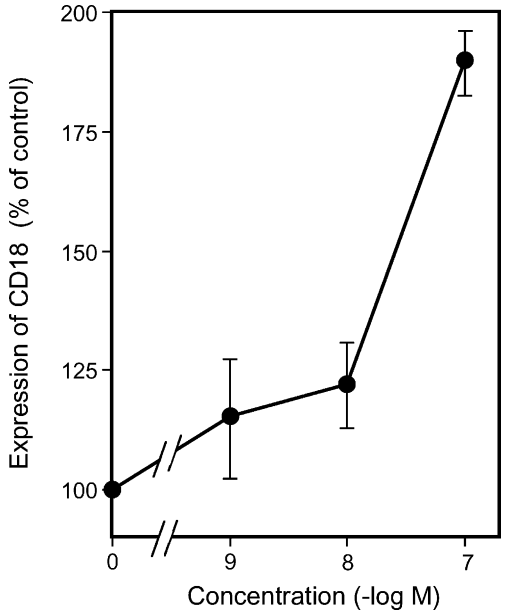


Fig. 2. Effect of TPT on CD18 expression on differentiated HL-60 cells. The cells were prepared as in Fig. 1, and CD18 expressed on the cell surface was determined by flow cytometry. Three independent experiments were performed, and the intensity of the fluorescence of 10^4 cells unexposed to TPT was normalized to 100%. The means and SDs are shown.

Fig. 3, a TPT-induced increase in CD18 mRNA was confirmed, and the mRNAs of G-CSFR, a neutrophil marker, and of p47phox and p67phox, components of superoxide-generating NADPH-oxidase [8], were also markedly increased by TPT. The other endocrine disruptors, including pesticides (DDT, atrazine, parathion, and vinclozolin), did not exhibit any such enhancing effect on gene expression. The data indicate that TPT has a unique and potent enhancing effect on the neutrophilic maturation of HL-60 cells among the endocrine disruptors tested thus far. We also employed a DNA macroarray derived from human immune cells to search for TPT-induced changes in gene expression (Fig. 4, upper table). The commercial array lacked the genes for p47phox and p67phox, but a TPT-induced increase in expression of G-CSFR was detected. TPT also augmented the expression of other functional phagocyte proteins, monocyte chemotactic protein 1 (MCP-1), CD14, and myeloid leukemia cell differentiation protein (MCL-1). NOTCH 1 has been thought to have functions that maintain hematopoietic cells in an undifferentiated state [14] and the TPT-induced decrease in NOTCH 1 corroborated the enhanced maturation of neutrophilic HL-60 cells. PTP also markedly decreased the expression of LC-PTP, a hematopoietic protein–tyrosine phosphatase. The results of real-time PCR clearly confirmed the unique suppressing effect of TPT on LC-PTP expression (Fig. 4, bottom). We then investigated the tyrosine-phosphorylating conditions in the TPT-treated cells.

TPT-enhanced expression of hck and components of NADPH-oxidase

Hck is a src-type tyrosine kinase that is more specifically expressed in neutrophils than other tyrosine kinases (reviewed in [15]). Hck consists of two splice-variant

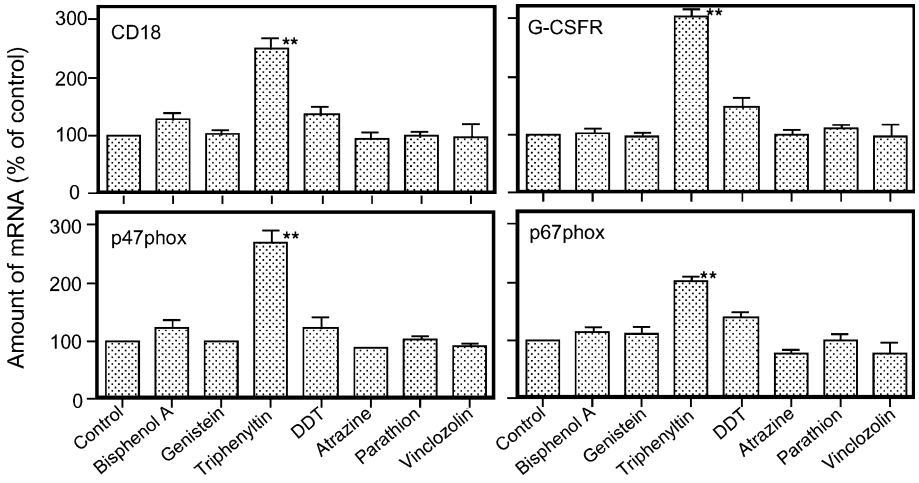


Fig. 3. TPT induced higher gene expression of neutrophil functional proteins than other endocrine disruptors. HL-60 cells were allowed to differentiate into neutrophils in the absence and presence of endocrine disruptors for 4 days, and the mRNAs of the proteins indicated were determined by real-time PCR. The endocrine disruptors used were 1 nM bisphenol A, 0.1 nM genistein, 0.1 μM TPT, 0.1 μM p,p'-dichrolodiphenyltrichloroethane (DDT), 10 nM atrazine, 1 nM parathion, and 0.1 μM vinclozolin. These concentrations were adopted as being the most effective concentrations for superoxide production by differentiated HL-60 cells. The mRNA levels of the proteins indicated derived from the cells differentiated without endocrine disruptors were normalized to 100% (control), and relative values of transcriptional expression are shown. The experiment was repeated three times and the means and SDs are expressed. $^{**}p < 0.01$.

forms (59 and 56 kDa), and since its gene is not on the commercial DNA array, we performed immunoblotting to investigate changes in the level of hck. As shown in

Name of Gene	Spot Intensity		Ratio	Up	Down
	Untreated	TPT-treated			
G-CSFR	46	81	1.76	1.8	
NOTCH1	253	147	0.58		1.7
MCP-1	72	125	1.74	1.7	
CD14	117	206	1.76	1.8	
LC-PTP	91	40	0.44		2.3
MCL-1	16	28	1.75	1.8	

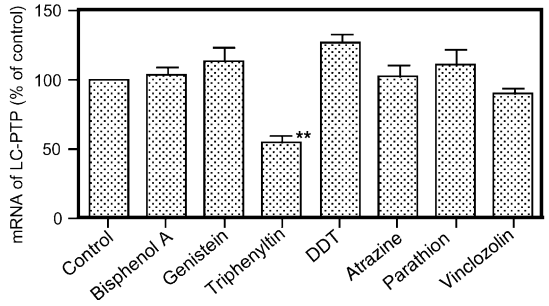
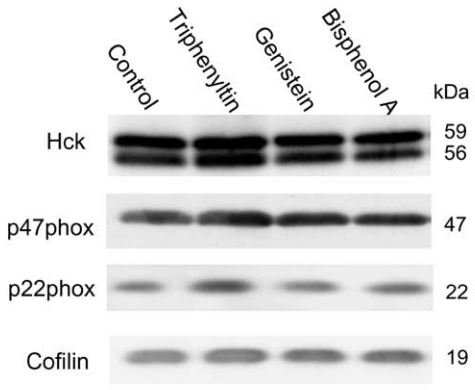


Fig. 4. Suppressive effect of TPT on gene expression of LC-PTP. *Top*: Results of DNA macroarray analysis of RNA derived from HL-60 cells differentiated in the presence of 0.1 μM TPT. MCP-1 and MCL-1 refer to monocyte chemotactic protein 1 and myeloid leukemia cell differentiation protein, respectively. *Bottom*: Real-time PCR analysis on LC-PTP. Experimental conditions were the same as in Fig. 3. The experiment was repeated three times and the means and SDs are expressed. $^{**}p < 0.01$.



		Control	Triphenyltin	Genistein	Bisphenol A
Hck	p59	100	130 **	107	96
	p56	100	110	106	104
p47phox		100	130 **	110	101
p22phox		100	197 **	119	134
Cofilin		100	108	110	103

(% of Control)

Fig. 5. Immunoblotting of hck, p47phox, p22phox, and cofilin. The proteins in the lysates of HL-60 cells differentiated in the absence (control) and presence of 0.1 μM TPT, 0.1 nM genistein, or 1 nM bisphenol A were detected by the specific antibodies and measured with image analyzers. *Photographs* (top): Typical staining patterns of the blotted membranes. *Table* (bottom): results of determination of each protein. Significantly different from the control (without TPT) $^{**}p < 0.01$.

Fig. 5, TPT augmented the expression of hck by about 30%. TPT also increased the expression of p47phox and p22phox, components of NADPH-oxidase. The enhancing effect was observed only with TPT and not with genistein and bisphenol A, the other endocrine disruptors tested. Cofilin is an actin-binding protein and is involved in superoxide production [11,12,16–20], but its expression level was unaffected by the endocrine disruptors.

Discussion

We investigated the effect of the environmental endocrine disruptor TPT on leukocyte differentiation in vitro and obtained several novel results. TPT displayed an enhancing effect on superoxide production by OZ-stimulated neutrophilic HL-60 cells at the low concentration of 0.1 μM, and the enhancement was accompanied by increased expression of CD18 (a subunit of the OZ-receptor), G-CSFR, and components of superoxide-generating NADPH-oxidase (p47phox, p67phox, and p22phox). There have been a few reports describing TPT as having a suppressive effect on neutrophil functions, including phagocytosis [5], chemotaxis [4], and superoxide production [3] in the micromolar concentration range. Marinovich et al. [6] recently described induction of apoptosis in HL-60 cells by exposure to TPT for 10 min at 5 μM. Our finding of enhancing effect at a lower dose of TPT was obtained by employing an HL-60 cell differentiation system and by characterization of the terminally differentiated neutrophilic cells after washing out TPT.

The mechanisms of TPT action remain unclear, however, there are hypotheses that organotin compounds inhibit the aromatase that catalyzes the change from androgen to estrogen or inhibits certain other steps in steroid metabolism [1] in invertebrates. In this paper we pointed out the possibility that a higher tyrosine-phosphorylation level may be involved in the effect of TPT on neutrophilic differentiation, because TPT lowered tyrosine phosphatase (LC-PTP) level and enhanced tyrosine kinase (hck) level. Our speculation is consistent with tyrosine phosphatase suppression of expression of the components of superoxide-generating NADPH-oxidase in neutrophils [21], involvement of tyrosine kinase in the activation of MAP kinase leading to respiratory burst [8], and tyrosine kinase involvement in OZ-triggered superoxide production [17]. TPT inhibited the activity of PU.1, a transcription factor involved in maturation of myeloid cells, during the differentiation of HL-60 cells (data not shown), which was the opposite of the effect of bisphenol A, which enhanced the activity of PU.1 [22]. TPT may affect other transcription factors that control the differentiation of neutrophils, such as NF-κB, C/EBPs, and AP.1, and since it is an organometal compound, TPT may also induce reactive oxygen intermediates (ROI) [5], which could activate NF-κB [23].

Organotin compounds have been detected in human organs, including the liver [24] and in blood [25], and TPT and tributyltin are the major active compounds [26]. Among various endocrine disruptors, organotin compounds might have a unique effect on innate immunity. To evaluate the low-dose effect of TPT we described above, in vivo studies are required.

Acknowledgments

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