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ESTROGEN MIMETIC 4-*tert*-OCTYLPHENOL ENHANCES IgE-MEDIATED DEGRANULATION OF RBL-2H3 MAST CELLS

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Allergic diseases such as asthma have been on the rise in recent decades. Environmental or occupational exposure to estrogenic synthetic chemicals is suspected to be a contributing factor, and previous experimental studies indicated that estradiol and some xenoestrogens increase allergic signaling responses, such as degranulation, in immune cells. In the current study, data showed that the estrogen mimetic 4-*tert*-octylphenol (4tOP) enhances immunoglobulin (Ig) E-mediated degranulation of mammalian mast cell line RBL-2H3 (RBL). At the noncytotoxic concentrations 10–20 μ M, 4tOP significantly increased degranulation in antigen (Ag)-activated RBLs but exerted no marked effect on spontaneous levels. Our data suggest that the industrial chemical 4tOP has the potential to enhance allergic disease in individuals who are exposed.

Worldwide, approximately 300 million people suffer from asthma (Masoli et al., 2004). Epidemiological investigations demonstrated a female bias in allergies and asthma that begins around puberty (McHugh et al., 2009; Vink et al., 2010). Along with evidence from hormone drug treatment studies (Barr et al., 2004), these human studies indicate that exposure to estrogenic chemicals promotes allergic disease.

Mast cells are effector cells in allergies and asthma. Immunoglobulin (Ig) E receptor cross-linking leads to signaling, influx of extracellular calcium, and degranulation/release of allergic mediators (Metzger et al., 1986). Mast cells also have receptors for estrogen. Zaitsev et al. (2007) reported that degranulation of the mast cell line RBL-2H3 is enhanced by

17 β -estradiol. Moreover, Narita et al. (2007) found that certain environmental estrogens similarly increase mast cell degranulation.

The alkylphenol 4-*tert*-octylphenol (4tOP) is used primarily in the manufacture of phenolic resins and is used, to a lesser extent, to produce alkylphenol ethoxylates in order to produce detergent surfactants (which degrade to their non-ethoxylated forms). 4tOP and its derivatives are used in many industries (CDC, 2009). Human biomonitoring studies revealed elevated levels of 4tOP due to occupational exposure (Chen et al., 2005).

In vitro, OP was found to bind estrogen receptors and alter transcription of estrogen-responsive genes (Bonefeld-Jorgensen et al., 2007; White et al., 1994; Mueller and Kim,

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1978). Exposure to 4tOP alters reproduction and sexual development in rats (Bian et al., 2006; Blake et al., 2004; Katsuda et al., 2000; Laws et al., 2000).

In the present study, the rat mast cell line RBL, which is functionally similar to human basophils (Metzger et al., 1986), was used to address whether the xenoestrogen 4tOP affects mast cell degranulation.

METHODS

Chemicals and Reagents

4tOP (CAS number 140-66-9) was obtained from Sigma-Aldrich (St. Louis, MO). Ethanol was ACS/USP grade from PHARMACO-AAPER (Brookfield, CT). Minimum essential media (MEM) with Earle's salts, without L-glutamine and phenol red, was from Cellgro (Manassas, VA); charcoal-dextran treated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

Cell Culture

Mast cells (RBL) were maintained as described (Palmer et al., 2012).

Preparation of 4tOP Stocks

4tOP stocks were made by dissolving granules in absolute ethanol, followed by vortexing and inverting before adding cell culture water (BioWhittaker Lonza, Walkersville, MD) to yield a vehicle of up to 60% ethanol (v/v). In assays, final concentrations of ethanol ranged from 0.12% to 0.6%.

Preparation of Cells for 4tOP Exposure

RBL were starved of hormones for 48 h before 4tOP exposure by being cultured in "steroid-stripped" RBL medium, a low-hormone media that contained phenol red-free MEM, 20% charcoal-dextran treated FBS, and 10 µg/ml gentamicin sulfate. After 24 h, cells were harvested and plated as described by Palmer et al. (2012). RBLs were then incubated

overnight in steroid-stripped media (Zaitsu et al., 2007).

Degranulation Assay

RBL to be stimulated to release the contents of their granules were pre-sensitized for 1 h with 0.1 µg/ml anti-dinitrophenyl (DNP) IgE (monoclonal, Sigma) that had been diluted in steroid-stripped media. Following this, a 1-h incubation with 4tOP or vehicle dissolved in bovine serum albumin (BSA)-Tyrodes (BT) buffer, ± DNP-BSA Ag, was performed (Palmer et al., 2012). Ag concentrations used in these experiments ranged from 0.00015 µg/ml to 0.01 µg/ml. Degranulation was quantified as before (Palmer et al., 2012).

Cytotoxicity Assays

To assess 1-h cytotoxicity of 4tOP in RBL, the release of lactate dehydrogenase (LDH) was measured with a kit from Roche (Palmer et al., 2012). Clonogenic cell survival cytotoxicity assays of 4tOP (24-h) were performed similarly to Palmer et al. (2012), with modifications: RBL were washed into steroid-stripped medium approximately 24 h before being plated. For the next 24 h, these plated cells were exposed to 4tOP or to vehicle alone (0.12% ethanol). After 24 h, medium was exchanged back to the full-hormone counterpart.

Statistical Analyses

Unless otherwise noted, significance was determined in Graphpad Prism software (San Diego, CA) using a one-way analysis of variance (ANOVA) followed by Tukey's test, as described in Palmer et al. (2012). The criterion for significance was set at $p < 0.05$. For Ag-stimulated degranulation, average percent degranulation was normalized to vehicle control before combining data from experiments, and comparisons were made to data from a low 4tOP dose (0.5 µM) that was never different from control. For spontaneous data, direct comparison to vehicle control was performed.

RESULTS

Inhibition of Degranulation in Hormone-Depleted Media

Compared with RBL bathed in complete media, hormone-starved RBL (cultured in steroid-stripped media) produce a less robust Ag-induced degranulation response (Table 1). While the maximal degranulation response in full-hormone media reached approximately 50% of intracellular granules released, this level is reduced to approximately 25% by culturing in steroid-stripped media. RBL degranulation was also suppressed in the presence of 0.6% ethanol, the vehicle for 4tOP administration (Table 1). This ethanol effect is in agreement with previous studies of Toviari et al. (2000).

4tOP Enhances Ag-Stimulated Degranulation

Figure 1A shows that 10 and 20 μ M 4tOP augment RBL degranulation by 1.3-fold and 1.4-fold, respectively, of the 0 μ M 4tOP (plus Ag) control levels. For these experiments, a range of Ag concentrations was utilized, and 4tOP consistently increases Ag-activated degranulation. Similar stimulatory effects of 4tOP were found when methanol was used as the vehicle for 4tOP (data not shown).

TABLE 1. Ag Dose Response for RBLs Cultured in Different Media

DNP-BSA (μ g/ml)	Full RBL media (avg % degran)	Steroid-stripped media (avg % degran)	Steroid-stripped media with ethanol (avg % degran)
0	2.4 $\pm$ 0.1	1.6 $\pm$ 0.4	1.8 $\pm$ 0.1
0.0001	14.1 $\pm$ 0.8	4.4 $\pm$ 0.6 (*)	1.4 $\pm$ 0.1 (*)
0.0004	37 $\pm$ 2	14.3 $\pm$ 0.7 (*)	7 $\pm$ 2 (*)
0.001	43 $\pm$ 3	15 $\pm$ 3 (*)	8 $\pm$ 2 (*)
0.01	48 $\pm$ 3	24 $\pm$ 3 (*)	16 $\pm$ 3 (*)

Note. Indicated media was present for the last 48 h before a 1-h exposure to DNP-BSA. The effect of the vehicle (0.6% ethanol) is also included. Values represent mean $\pm$ SD of triplicate samples for representative experiments. Statistical significance in comparison to full RBL media was determined by two-way ANOVA with a Bonferroni post-test, and is denoted by (*) $p < 0.05$. Avg, average; degran, degranulation.

As seen in Figure 1B, 4tOP neither elevates nor decreases spontaneous levels of degranulation from non-stimulated cells (no IgE, no Ag). An assay for degranulation effects after overnight 4tOP exposure revealed similar ability to increase Ag-stimulated degranulation (data not shown).

4tOP Doses That Enhance Degranulation Do Not Cause RBL Cell Death

In cytotoxicity assays designed to replicate conditions in the degranulation experiments (up to 20 μ M 4tOP), no change was found in the percentage lactate dehydrogenase (%LDH) released from cells that were treated with 4tOP (Figure 1C). In addition, no difference was found in the ability of RBL to grow colonies after 24-h 4tOP exposure (Figure 1D). Thus, 4tOP concentrations used in the degranulation experiments did not affect cell viability.

DISCUSSION

The key finding of this study is that the xenoestrogen 4tOP enhanced Ag-stimulated RBL mast cell degranulation, an important physiological process in allergic disease. Further, a suppressive effect of steroid-stripped media on antigen-induced RBL degranulation is documented here. Thus, it is clear that at least some of the many hormones and other small biomolecules that are depleted in the charcoal stripping process (Cao et al., 2009) are important for robust mast cell functioning.

This study supports the hypothesis that enhanced mast cell degranulation is a route by which estrogenic chemicals contribute to the severity of allergic diseases. These new 4tOP data concur with previous data showing that several other estrogenic compounds (estradiol and xenoestrogens) promote mast-cell degranulation (Zaitsu et al., 2007; Narita et al., 2007). In future studies, the mechanism underlying this effect will be more closely evaluated. It is postulated that 4tOP stimulates calcium signaling (and, thus, degranulation) by binding and activating a membranous estrogen

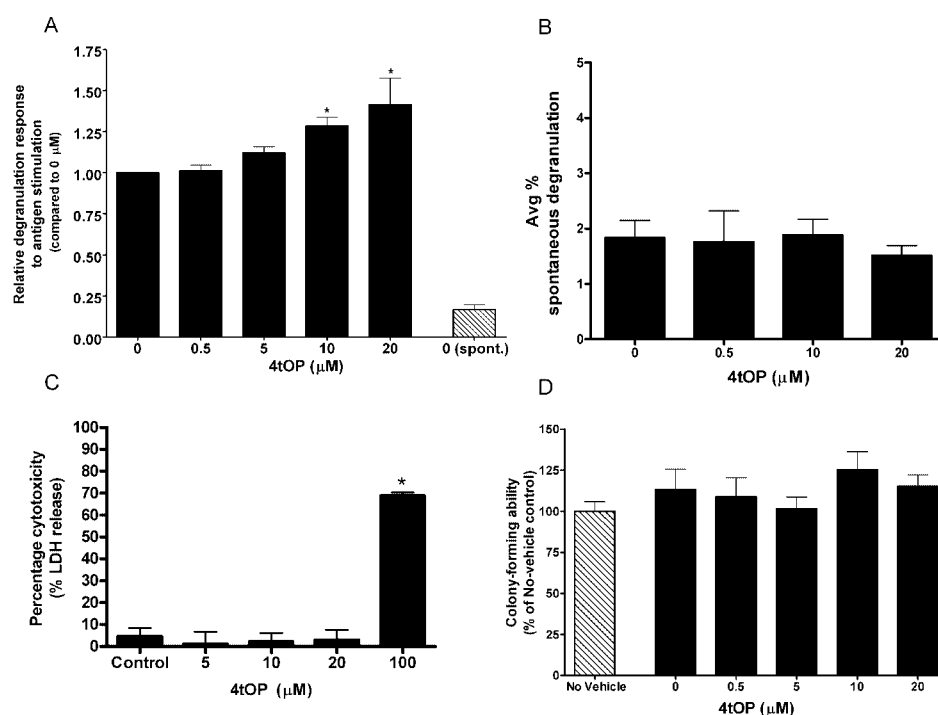


FIGURE 1. (A) IgE-sensitized cells were stimulated with Ag for 1h in the presence of 4tOP. Normalized degranulation response is plotted against 4tOP doses. An average spontaneous release value, normalized to Ag-stimulated control, is depicted for reference. Values represent the mean $\pm$ SEM of 6–19 independent experiments; 3 replicates were performed for each experiment. Significance indicated by * $p < 0.05$. (B) Spontaneous release during 1h (no IgE or Ag), where average percent degranulation as a raw value is plotted against 4tOP dose. Values represent the mean $\pm$ SEM for 17 independent experiments. (C) Cytotoxicity to 4tOP (1h) was assessed by an LDH assay from Roche. Values represent means $\pm$ SEM of two to six separate experiments, each performed in triplicate. Significance indicated by * $p < 0.05$. (D) The 24-h cytotoxicity to 4tOP assessed by a clonogenic colony-forming assay, where values represent mean of 2 experiments (3 replicates each), $\pm$ SEM. Each experiment was normalized to the mean control value, which was set at 100%.

receptor, as was shown for estradiol (Zaitsev et al., 2007). These data call attention to an allergic response that may affect those occupationally or environmentally exposed to 4tOP.

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