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Tributyltin or Triphenyltin Inhibits Aromatase Activity in the Human Granulosa-like Tumor Cell Line KGN

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The superimposition of male sex organs (penis and vas deferens) in a female gastropod, called imposex, is widely attributed to the exposure to tributyltin (TBT) compounds, used world-wide in antifouling paints for ships. It has been hypothesized that the TBT-induced imposex is mediated by an increasing androgen level relative to the estrogen level, namely a decreased conversion of androgens to estrogens (i.e., aromatization). In the present study, we tested this hypothesis by examining the effects of TBT or triphenyltin (TPT) on the aromatase activity in a cultured human granulosa-like tumor cell line, KGN, which was recently established by our group. Treatment with more than 1000 ng/ml TBT compounds was very toxic to the cells and caused immediate cell death within 24 h, while 200 ng/ml was found to cause apoptosis of the cells. Treatment of the KGN cells for more than 48 h with 20 ng/ml TBT or TPT, which is a concentration level reported to cause imposex in marine species, did not affect cell proliferation but significantly suppressed the aromatase activity determined by a $[^3H]H_2O$ release assay. Treatment with 20 ng/ml TBT compounds for 7 days also resulted in a reduction of the E2 production from Δ4-androstenedione stimulated by db-cAMP. The changes in the aromatase activity by TBT compounds were associated with comparable changes in P450arom mRNA assessed by RT-PCR. The luciferase activity of the P450arom promoter II (1 kb) decreased after the addition of 20 ng/ml TBT compounds in transfected KGN cells either in a basic state or in states stimulated by db-cAMP. The Ad4BP-dependent increase in the luciferase activity of P450arom promoter II was also downregulated by such treatments. These results indicate that TBT compounds inhibited the aromatase activity and also decreased the P450arom mRNA level at the transcriptional level in KGN cells. The direct inhibitory effect of TBT compounds on the aromatase activity may therefore partly explain the induction of imposex by these compounds in female species. © 2001 Academic Press

Key Words: **granulosa-like tumor cell; KGN; aromatase; tributyltin; triphenyltin; imposex.**

The use of tributyltin (TBT) as biocides in antifouling paints and wood preservatives leads to the contamination of the marine and freshwater environment. TBT has been shown to be highly toxic to a number of aquatic animals. Especially in marine prosobranch snails, TBT induces reproductive abnormalities and sterilization in female animals. This phenomenon, which has been called either pseudohermaphroditism or imposex, is characterized by the development of additional male sex organs (penis and/or vas deferens and prostate tissue) on females (1, 2). Not only TBT but also triphenyltin (TPT) has been shown to have a strong effect on the development of imposex in the rock shell, *Thais clavigera* (3). However, the detailed biochemical mechanism of this phenomenon remains obscure. The involvement of sex steroids in the expression of imposex of marine neogastropods has been

Abbreviations used: P450arom, cytochrome P450 aromatase; TBT, tributyltin; TPT, triphenyltin; FCS, fetal calf serum; DCS, dextran-coated, charcoal-treated FCS; db-cAMP, dibutyryl cyclic AMP; RT-PCR, reverse transcriptase–polymerase chain reaction; PBS, phosphate-buffered saline; RIA, radioimmunoassay; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CREB, cAMP-regulatory element binding protein.

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suggested based on the fact that pure female displays the lowest testosterone content, whereas advanced imposex stages have the highest testosterone content (4) as well as the fact that the TBT-induced imposex was completely suppressed by antiandrogen cyproterone acetate which is a competitive inhibitor of androgen receptors (4). The TBT-induced imposex is mediated by an increasing androgen level relative to the estrogen level, thus suggesting a decreased conversion of androgens to estrogens (i.e., aromatization) (4, 5). This has been further supported by the fact that a specific aromatase inhibitor, SH489 showed the same imposex-inducing effect as a TBT-exposure did in neogatropods (4). On the other hand, there is a controversial report which demonstrated no decrease in the activity of aromatase in the gastropods, which were contaminated and exhibited clear evidence of imposex (6). To our knowledge, no clear *in vitro* direct evidence has yet demonstrated whether or not TBT compounds truly affects the aromatase activity in any species including marine species as well as in humans.

We recently established a steroidogenic human ovarian granulosa-like tumor cell line from a patient with invasive granulosa cell carcinoma (7). The cell line possesses properties very similar to normal ovarian granulosa cells, including the expression of functional FSH receptor and a relatively high aromatase activity. The cell line is, thus considered to be a very useful model for investigating the *in vitro* effects of various compounds on the aromatase activity in mammalian system. In this study, to clarify the mechanism of female muscularization by organotin compounds, we attempted to investigate whether TBT compounds may have a direct effect on the aromatase activity in a mammalian system using this useful cell line.

MATERIALS AND METHODS

Materials. Tributyltin (TBT) and Triphenyltin (TPT) were obtained from the Sigma Chemical Co. (St. Louis, MO). Both compounds were dissolved in ethanol, and the final concentration of ethanol in the cell growth medium was 0.1% (v/v). A specific aromatase inhibitor, YM511 was kindly provided by Yamanouchi Pharmaceuticals (Tokyo, Japan) (8). DMEM/F12 and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY). [β-^{3}H]Androstenedione was purchased from Amersham Pharmacia Biotech (Boston, MA). *Taq* DNA polymerase, pGEM-T-easy vector, pGL3 luciferase reporter vector and Dual-luciferase reporter assay system were obtained from Promega (Madison, WI). The plasmid DNA isolation kit and SuperFect transfection reagents were purchased from Qiagen (Hilden, Germany). All of the primers for PCR were synthesized by Amersham–Pharmacia Biotech (Osaka, Japan). Dibutyryl cyclic AMP (db-cAMP) was purchased from Sigma Chemical Co.

Cells and cell growth assay. A human ovarian granulosa-like tumor cell line, KGN was recently established by us (7). Briefly, the cells were obtained from a 63-year-old female patient with invasive granulosa cell carcinoma. The cells possess properties similar to those of normal granulosa cells, including the expression of functional FSH receptor and a relatively high aromatase activity as well as 17β-HSD activity. This cell line can thus produce estrogens like estrone (E1) and estradiol (E2) in the presence of exogenous substrate, 4-androstene-3,17-dione. The cells were maintained in DMEM/F12 supplemented with 10% FCS in an atmosphere of 5% CO_2 at 37°C.

For growth experiments, the cells were plated onto a 24-well plate at 1×10^4 cells/well in DMEM/F12 supplemented with 10% FCS. The cells were treated with TBT or TPT, while control cells were treated with ethanol alone, and the medium with drugs or ethanol was changed every 2 days. After washing, the cells were trypsinized and then counted using a hemocytometer every 48 h for 7 days. Cell viability was assessed by the trypan blue exclusion method.

Detection of apoptosis by fluorescence microscopy. Early and late apoptotic changes in human granulosa cells were also determined using an Annexin V-EGFP/PI apoptosis detection kit as previously described (9). One day before treatment, the cells were divided into 35-mm glass-bottom dishes (MatTek Corp.) and then were treated with 200 ng/ml TBT for 48 and 72 h, respectively. After the treatment, the cells were washed once with PBS and then were incubated with 200 μl 1× binding buffer containing 1 μl annexin V-EGFP and 1 μl propidium iodide (PI) at room temperature for 5 min in the dark. The cells were then scanned using confocal laser scanning microscopy (Leica TCS-SP system, Leica Microsystems, Heidelberg, Germany) using a dual filter set for FITC and TRITC. The cell membrane was imaged for green fluorescence (stained by annexin V-EGFP) by excitation with the 488-nm line from an argon laser, and the emission was viewed through a 460- to 505-nm band pass filter. The cell nucleus was imaged for red fluorescence (stained by PI) by excitation with the 560-nm line from an argon laser, and then the emission was viewed through a 520- to 580-nm band pass filter.

Aromatase assay. The aromatase activity was determined by measuring the [^{3}H]H_2O release upon the conversion of [1β-^{3}H]androstenedione (A) to estrone (E1), as described previously (10). Before the experiment, the cells were cultured in DMEM/F12 with 5% DCS (dextran-coated, charcoal-treated FCS) for 48 h. After the cells were treated with either TBT or TPT, [1β-^{3}H]androstenedione was added, and then the cells were further incubated for 6 h. The medium (2.0 ml) was extracted with chloroform and then was centrifuged. The aqueous supernatant was mixed with 5% charcoal/0.5% dextran and then was incubated for 30 min. Thereafter, the mixture was centrifuged and the supernatant was added to 5 ml of scintillation fluid and assayed for radioactivity. The amount of radioactivity in [^{3}H]H_2O thus measured was standardized based on the protein concentration which was determined using a micro BCA kit (Pierce Chemical Co., Rockford, IL) and expressed as pmol/mg protein/6 h.

Estradiol content assayed by RIA. To ensure that the measured aromatase activity truly reflected the capability of estrogen production, the cells were treated with or without various concentrations of TBT or TPT for 7 days, and then were further incubated with 4-androstene-3,17-dione (1 μM) for 12 h. The medium was collected and the E2 (estradiol) content in the medium was determined by specific RIA (SRL, Inc., Tokyo, Japan).

RNA extraction and RT-PCR analysis. RNA extraction and RT-PCR analysis for aromatase mRNA were performed as described previously by us (11).

Plasmid construction and luciferase analysis. To further determine whether or not the decreased expression of P450arom mRNA was regulated through promoter II, which is the major promoter in ovarian granulosa cells (7, 12, 13), luciferase activity was determined using a 1-kb P450arom promoter II expression construct in pGL3 basic vector (14). Transfection was performed using SuperFect reagents according to the manufacturer's instructions. Briefly, the cells (1×10^5) were seeded in a six-well plate 12 h prior to transfection, and they were transfected with 2 μg DNA. Five nanograms of *Renilla* luciferase control reporter pRL-CMV, as an internal standard, was added per well to assess the transfection efficiency. On the day after

transfection, the cells were treated with or without TPT or TBT. The cells were maintained at 37°C for 48 h and then were lysed and harvested and thereafter were subjected to a luciferase analysis using the Dual-luciferase reporter assay system according to the manufacturer's instructions.

Statistical analysis. Differences between the aromatase activity, E2 values and P450arom mRNA for control cells and cells treated with the drugs were determined by Student's *t* test. $P < 0.05$ was considered statistically significant. All data points are presented as means ± SD.

RESULTS

The Concentration of TBT or TPT at 20 ng/ml or Less Had No Effect on KGN Cell Growth

Since TBT or TPT is well known to be toxic to several cells or to cause apoptosis (15), it is necessary to determine the effect of TBT or TPT on KGN cell growth. More than 1000 ng/ml of TBT or TPT were observed to be very toxic to KGN cells and all of the cells died within 24 h (data not shown). At a concentration of 200 ng/ml of TBT for 48 h, cell proliferation was suppressed to almost 50% of untreated cells, and some apoptotic cells were observed by Annexin V-EGFP/PI staining (Fig. 1). Annexin V binds with a high affinity to negative charged PS, and it has been used in combination with PI to detect early and late apoptotic or necrosis cells. In unexposed control cells, most cells stained for neither annexin V-EGFP (green) nor PI (red). After being treated with 200 ng/ml TBT, early apoptotic cells with a cell membrane stained by green color (annexin V positive/PI negative) were detected at 48h, and late apoptotic cells with a cell membrane demonstrating green staining and red staining for nuclear staining (annexin V positive/PI positive) were seen at 72 h (Fig. 1). However, TBT or TPT at concentrations of less than 50 ng/ml (2 or 20 ng/ml) had little effect on the KGN cell proliferation for 7 days (data not shown). As a result, a concentration of 20 ng/ml of TBT or TPT, which was reported to induce imposex in marine species, was used in the following experiment.

TBT or TPT Inhibits Aromatase Activity

To determine the regulation of aromatase activity in KGN cells, we first incubated the cells with 20 ng/ml TBT or TPT for 72 h in the presence or absence of db-cAMP, and then aromatase activity was assessed by $[^3H]H_2O$ release assay. The basal level of the aromatase activity in KGN cells was 0.435 ± 0.041 pmol/mg protein/6 h, and TBT or TPT slightly but significantly inhibited the aromatase activity to about 30% of the baseline ($P < 0.05$) (Fig. 2A). While 10^{-4} M db-cAMP treatment gave rise to a 1.7-fold increase of the aromatase activity over baseline, the increase was also suppressed by 20 ng/ml TBT or TPT (Fig. 2A). The time course for the effect of 20 ng/ml TBT on the basal or db-cAMP-stimulated aromatase activity was investigated over a 7-day period (Fig. 2B). A significant inhibition of the aromatase activity was observed either in a basic state or in states stimulated by 10^{-4} M db-cAMP, respectively, as early as 3 days after the addition of the drugs and a maximal suppression was observed at day 7 (Fig. 2B). The aromatase activity determined by a $[^3H]H_2O$ release assay did not definitively verify the capability of estrogen production. We next assessed the db-cAMP-stimulated E2 production in the cells with or without TBT or TPT treatment of for 7 days. The treatment with 20 ng/ml TBT or TPT for 7 days caused a significant decrease in the E2 concentration in the medium, which was consistent with the changes observed in the aromatase activity (Fig. 2C).

TBT Decrease P450arom mRNA

To investigate whether or not the changes in the aromatase activity in KGN cells were associated with comparable changes in the levels of P450arom mRNA, total RNA was extracted from cells maintained in the absence or presence of 20 ng/ml of TBT for 48 h. The mRNA expression levels of P450arom relative to that of β-actin were shown in Fig. 3. Changes in the P450arom mRNA levels were associated with comparable changes in the aromatase activity. Therefore, the decrease in the aromatase activity of KGN cells treated with TBT was related to the decrease in the P450arom mRNA level.

Luciferase Activity

The promoter activity was determined and shown as a fold-increase in the luciferase activity normalized for *Renilla* luciferase activity (termed relative luciferase activity). The luciferase reporter driven by P450arom promoter II displayed a 100-fold higher luciferase activity compared with the empty vector, pGL3-basic (data not shown). As shown in Fig. 4, 10^{-4} M db-cAMP induced a 1.7-fold activation of the promoter. The luciferase activity was decreased by the addition of TBT compounds in transfected cells either in a basic state or in states stimulated by cAMP, respectively. In addition, the forced expression of Ad4BP/SF-1 caused an 8-fold activation of the promoter. The Ad4BP/SF-1-dependent increase of the luciferase activity was also significantly suppressed by both compounds.

DISCUSSION

In females, estrogens are mainly synthesized in ovarian granulosa cells before menopause and are produced by the conversion of androgens. The biosynthesis of estrogens from androgens is catalyzed by an enzyme complex which has been called aromatase (*CYP 19,*

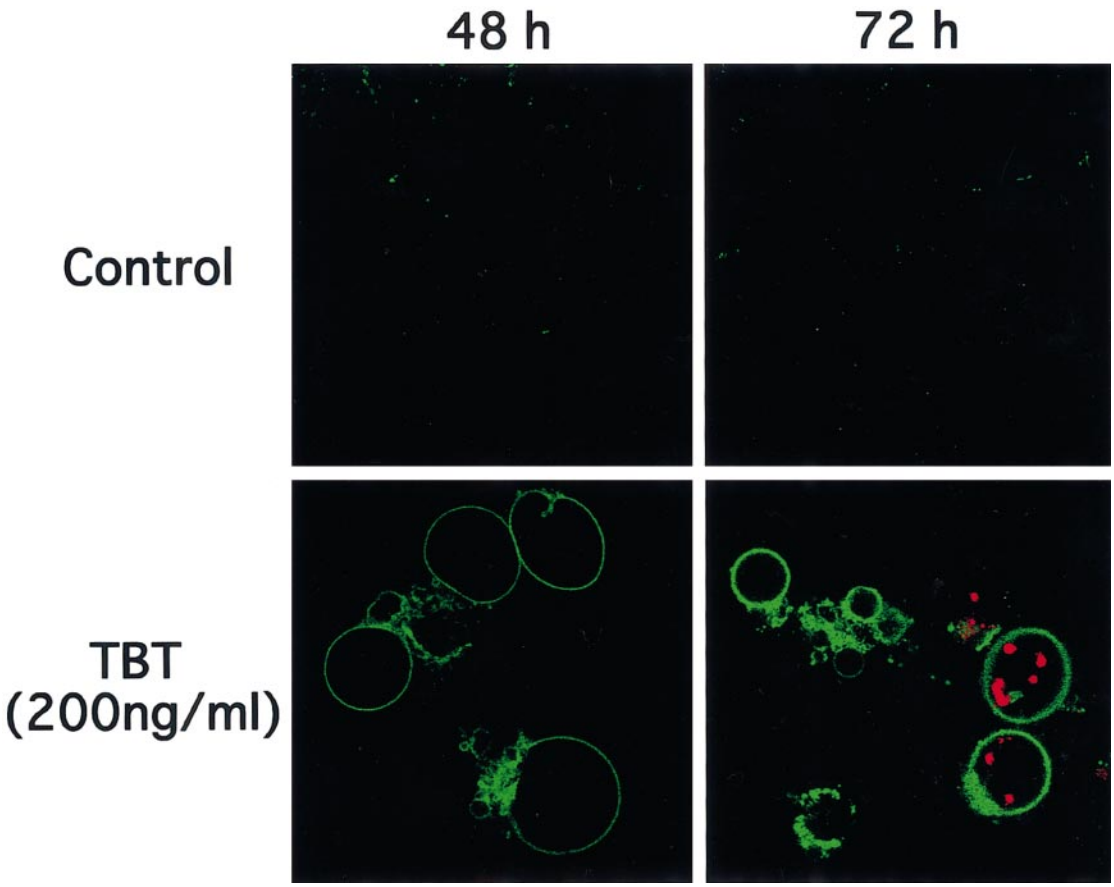

FIG. 1. Morphological evidence for apoptosis induced by TBT. After incubating the granulosa cells with 200 ng/ml TBT for 48 and 72 h indicated, the cells were stained by annexin V-EGFP and propidium iodide as described under Materials and Methods. The cells were then scanned using a confocal laser scanning microscopy. The early apoptotic cells with the cell membrane stained by annexin V-EGFP (green) at 48 h and the late apoptotic cells with both the cell membrane stained by annexin V-EGFP and nucleus stained by propidium iodide (red) at 72 h were detected.

cytochrome P450arom) (16). P450arom is present in many tissues, including the gonads, brain, placenta, bone and adipose tissue (16–19). The tissue-specific expression of the P450arom gene is mediated by tissue-specific promoters using alternative splicing of exon 1 (20, 21). In ovarian granulosa cells, promoter II in the *CYP 19* gene is mainly utilized for its transcriptional regulation. In addition, the transcriptional regulation of the P450arom gene in ovarian granulosa cells has been demonstrated to be activated by the cAMP–protein kinase A pathway and is also dependent on a steroidogenic tissue-specific transcriptional factor, Ad4BP/SF-1 (13, 22). In addition, it is reported that cooperative or additive interaction between Ad4BP and CREB is required for cAMP activation of promoter II in granulose cells (13).

In the present study, we demonstrated that the treatment with TBT or TPT directly inhibits the aromatase activity in a cultured human granulosa-like tumor cell line, KGN cells either at a basic state or in the states stimulated by db-cAMP. This finding was further supported by the actual decrease of E2 concentration in the cultured medium. The inhibitory effect of aromatase activity by TBT or TPT in KGN cells was clearly not due to the competitive inhibition of P450arom because the inhibitory effect by TBT or TPT was very slow to take place (more than 48 h), which was a striking contrast to a rapid (within 5 min) and complete suppression of the aromatase activity by a competitive aromatase inhibitor, YM511 (8) in the same culture system (data not shown). The changes in the aromatase activity by TBT compounds were associated with comparable changes in the P450arom mRNA level as assessed by RT-PCR. In addition, the luciferase activity of P450arom promoter II (1 kb) decreased after the addition of compounds in transfected KGN cells either at a basic state or in the states stimulated by db-cAMP. An Ad4BP/SF-1-dependent increase of the luciferase activity of P450arom promoter II was also downregulated by such treatments. Based on the above findings, TBT-induced suppression of aromatase activity appears to be partly regulated at the transcriptional level in association with the cAMP–PKA pathway or regulation by Ad4BP/SF-1. However,

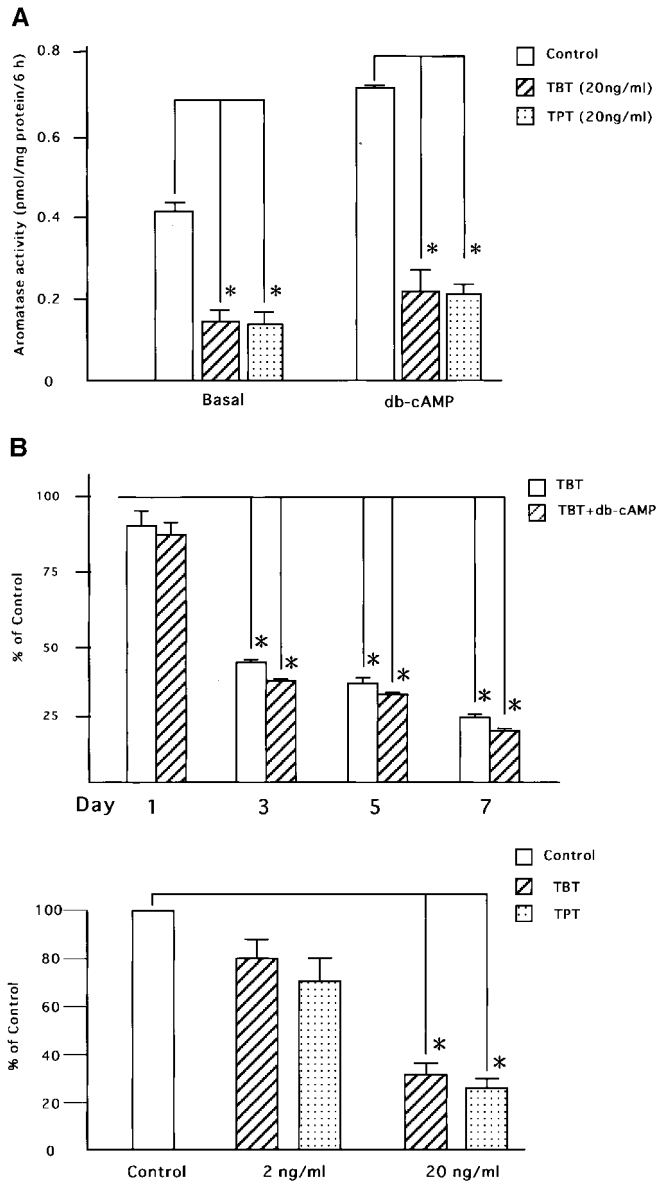

FIG. 2. Effect of TBT or TPT compounds on the aromatase activity in cultured KGN cells. (A) Regulation of the aromatase activity in cultured KGN cells. The granulosa cancer cells, KGN cells were treated with 20 ng TBT or TPT for 48 h in the presence or absence of 10^{-4} M db-cAMP. The method of determining the aromatase activity was described under Materials and Methods. Data represent means ± SD from three independent experiments done in triplicate. The aromatase activity was standardized based on the protein concentration and is presented as pmol/mg protein/6 h. *$P < 0.01$ vs control cells treated with ethanol. (B) The time course for the effect of 20 ng/ml TBT on the basal or 10^{-4} M db-cAMP stimulated aromatase activity was investigated over a 7-day period. Data represent means ± SD. The results were representative of three independent experiments done in triplicate. When the aromatase activity of control cells was expressed as 100%, the relative aromatase activity treated with 20 ng TBT or TBT + 10^{-4} M db-cAMP was expressed as percentage of control. *$P < 0.01$ vs control cells treated with ethanol. (C) Effect of TBT or TPT compounds on the E2 production in cultured KGN cells. The cells were treated for 7 days with 2 or 20 ng/ml TBT or TPT. E2 production was assayed by RIA. Data represent means ± SD from three experiments done in triplicate. *$P < 0.05$ vs control cells treated with ethanol.

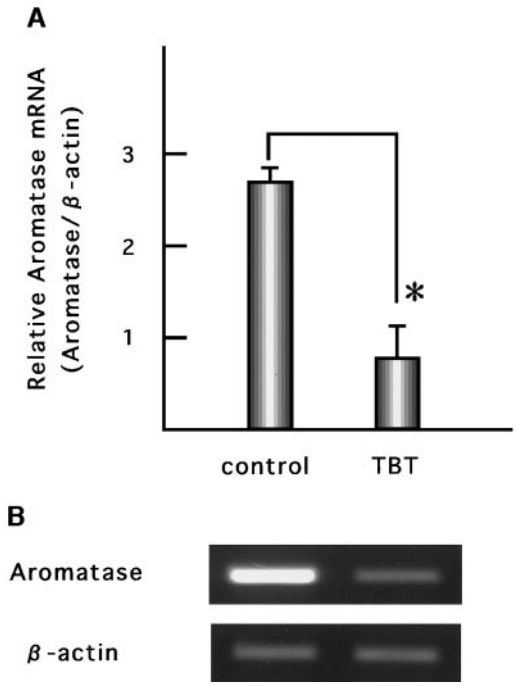

FIG. 3. Effect of TBT compounds on expression of P450arom and β-actin transcripts in cultured KGN cells. Total RNA was extracted from cells maintained in the absence or presence of 20 ng/ml of TBT for 48 h. (B) The RT-PCR products were electrophoresed on 2% agarose gel containing 0.5 mg/ml ethidium bromide. (A) The relative expression levels of P450arom mRNA were determined by the measuring of the intensity of the ethidium bromide. Data represent means ± SD from three experiments done in triplicate. *$P < 0.05$ vs control cells treated with ethanol.

at least, the expression level of Ad4BP determined by RT-PCR was unchanged by the treatment with TBT or TPT (data not shown). Further studies are needed to elucidate a more precise mechanism.

The dose ranges of TBT or TPT that inhibited the aromatase activity, were to 2–20 ng/ml (corresponding to 0.6–6 nM), which are almost pharmacologically relevant to the ranges which are reported to induce imposex in female gastropod (1–3). While admittedly the species are different, our findings may support the previously reported interesting hypothesis that TBT compounds may disturb the aromatase activity, thus leading to the induction of imposex in female aquatic animals (1–5). This also suggests possibility that long-term and excessive exposure to TBT compounds in humans may also cause some clinical problems relating to the excessive androgens and/or reduced estrogens, especially in women, such as ovarian dysfunction, osteoporosis and hirsutism. Induction of apoptosis of granulosa cells at higher concentrations of TBT compounds may also indicate a possibility that a more TBT-polluted environmental situation could cause much more severe ovarian dysfunction.

Another explanation for imposex by organotin compounds in gastropods was suggested to be the

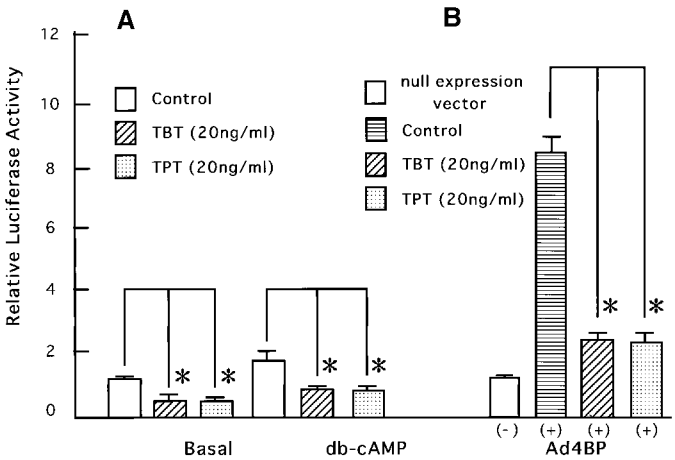

FIG. 4. TBT or TPT decreases the luciferase activity of P450arom promoter II in granulosa cancer cells. (A) Fusion gene construct containing a 1-kb of P450arom promoter II was transfected into KGN cells, and then the 20 ng/ml of TBT or TPT was added to the medium in the presence or absence of 10^{-4} M db-cAMP for 48 h. *$P < 0.01$ vs control cells treated with ethanol. (B) Fusion gene construct containing 1 kb of P450arom promoter II was also cotransfected into KGN cells with bovine Ad4BP expression vector, RSV/Ad4BP or a null expression vector as a negative control. Data represent means ± SD from three experiments done in triplicate. * $P < 0.01$ vs control cells treated with ethanol. Other details are described under Materials and Methods.

inhibition of androgen excretion due to a decrease in the sulfur conjugation of androgen, although this mechanism remained unclear (23). In addition, as a third attractive explanation, it was recently reported that TBT and TPT are potential activators of androgen-receptor-mediated transcription in mammalian cells (24). Therefore, the mechanism of imposex by organotin may be attributed not simply to aromatase inhibition but may also be related to several other factors including a decrease in sulfur conjugation of androgen and upregulation of AR-mediated transcription. This idea is probably reasonable because each reported mechanism itself does not seem to be strong enough to explain such a dramatic sex reversal like imposex.

In summary, we demonstrated that TBT or TPT inhibits aromatase activity in the *in vitro* mammalian system using human granulosa cancer cells KGN, partly at the transcriptional level. This mechanism may therefore partly explain the occurrence of organotin-induced imposex.

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