nancy. Thus such endocrine functions of placenta might be at high risk due to the developmental and reproductive toxicity of environmental contaminants which have endocrine-disrupting effects. Previously, we reported that TBT and TPT enhance hCG secretion and aromatase activity in human choriocarcinoma cells (J. Clin. Endocrinol. Metab. 87, 2830, 2002). In the present study, in order to extend the knowledge on correlation between the structure of organotin compounds and their endocrine-disrupting effects, we assessed effects of their various alkyl derivatives on placental hCG secretion and aromatase activity of human choriocarcinoma Jar cell, suggesting that the potency of disrupting effects induced by organotin compounds has relationships to both the number and the length of their side chain with regularity.

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## INFLUENCE OF PHYTOESTROGENS ON AROMATASE ACTIVITY AND ESTROGEN-DEPENDENT CELL GROWTH

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Approximately 60% of all mammary carcinoma cells, the most common female cancer, grow in an estrogen-dependent fashion. Estrogen can be synthesized by tumour cells themselves from precursors with aromatase being the key enzyme involved. Consequently, estrogen positive mammary carcinomas are treated with anti-estrogens to slow further tumour growth. Epidemiological evidence indicate a correlation between breast cancer risk and the consumption of specific nutrients rich in phyto estrogens like soy beans. Estrogen formation is affected by a variety of environmental chemicals, prominent among them plasticizers like 2-ethylhexyl phthalate, and herbicides. Plant ingredients affecting estrogen formation are summarily called phytoestrogens, and have been detected e.g. in leguminoses like peas and soy beans. Isoflavones, coumestans and lignans are estrogenic or antiestrogenic, in a concentration dependent fashion. Other plants are poorly investigated, estrogenic or antiestrogenic activity has been detected in a variety of species including mulberry trees, cactus flowers or tobacco. Therefore it seems reasonable to assume that phytoestrogens are present in many plants and will be active at relevant concentrations. No studies have yet been conducted investigating aromatase effects from medical plants.

Besides direct interaction with estrogen formation or estrogen receptor binding, breast cancer growth may be affected by interference with intracellular estrogen signal transduction or nuclear activity interference; for genistein, a soy bean phytoestrogen, these effects have been postulated. We will present preliminary results for plant ingredient effects on aromatase activity as well as a summary of known and possible interactions between aromatase activity and plant extracts from food and medicinal sources.

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## ALTERED GENE EXPRESSION OF NITRIC OXIDE SYNTHASE IN THE RAT PLACENTA EXPOSED TO 3,3′,4,4′, 5-PENTACHLOROBIPHENYL

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We have found that exposure to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induces CYP1A1 in the fetal capillary endothelial cells in the labyrinth zone of the rat placenta. The induction of CYP1A1 indicates that PCB126 exposes fetal endothelial cells and binds to aryl hydrohydrocarbon receptor in these cells. However, the effect of PCB 126 on the placental function has not been evaluated. Among many important factors for the placental development and function, nitric oxide generated by its synthase (NOS) might play a role in the maintenance of the placental function. In the present study, we examined the gene expression of two isoforms of NOS (iNOS and eNOS) to asses the effects of PCB126 on the rat placenta. The pregnant Sprague-Dawley rats were given a single oral dose of 100 μg PCB 126/kg body wt or an equivalent volume of corn oil (control) on gestational day (GD) 18 and their placentas were collected on GD 20. The placental specimens were used for quantitative analysis of the gene expression of eNOS, iNOS and CYP1A1 by real-time PCR. The number of the placenta with live fetus did not significantly differ between control and PCB-exposed groups. Quantitative RT-PCR analysis showed significantly decreased expression of eNOS and obvious expression of CYP1A1 in the PCB-exposed placenta. Also the level of the expression of iNOS in the PCB-exposed rats was lower than that of controls. These results indicate that PCB126 alters gene expression of NOS in the rat placenta.

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## IN VITRO ASSAYS FOR SCREENING OF ENDOCRINE DISRUPTORS: RELATIONSHIPS BETWEEN HUMAN ESTROGEN RECEPTOR BINDING ASSAY AND REPORTER GENE ASSAY ON 948 CHEMICALS

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Bindings of chemicals to estrogen receptor (ER) followed by gene transcriptional regulation are known as one of the key modes of actions that may disrupt the endocrine system. Both in vitro receptor binding and gene transcriptional assays are simple and valuable to screen such endocrine disrupting chemicals from numerous chemicals produced. To utilize each in vitro assay practically, it is important to confirm the relationships between assays and evaluate the validity of each assay. In this study, ER-binding affinities and ER-mediated transcriptional activities of approximately 948 chemicals were measured and the relationships between the two assays were evaluated. The test chemicals were selected to encompass diverse chemical structures from commercially available reagents with molecular weight of 100 to 600, excluding inorganic compounds. Competitive binding assay was performed using recombinant human ER alpha ligand binding domain expressed in *E. coli* and tritium-labeled 17beta-estradiol (E2) as a tracer. Relative binding affinity (RBA), which was a percent ratio of IC50 values of E2 and test chemicals, was employed for comparison of the receptor-binding potency among chemicals. HeLa cell stably co-transfected with receptor expression and reporter plasmids was used for reporter gene assay. Transcriptional activity of chemical was designated by a half-potent concentration (PC50), which defines as a concentration of test chemical activating at 50% of maximal transcriptional activity by E2. As the results, 168 chemicals showed both ER-binding and ER-mediated transcriptional potencies in these assays, and RBA and PC50 values in most of these chemicals exhibited good relationships between two in vitro assays. Some outliers from this correlation were observed and they included characteristic chemicals, which had ester bond in their molecules or presumably behaved as antagonists based on their structures.

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# Trialkyltin Compounds Enhance Human CG Secretion and Aromatase Activity in Human Placental Choriocarcinoma Cells

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**Human choriocarcinoma cell lines have been used as placental models for the study of endocrine function, including aromatase (CYP19) activity and the secretion of human CG (hCG). In the present study, we investigated the effects of trialkyltin compounds, which are suspected endocrine disrupters, on aromatase activity and hCG secretion in human choriocarcinoma JAR, JEG-3, and BeWo cells. Protein synthesis as measured by $^{35}$S-methionine incorporation in all cell lines was markedly decreased by treatment with both tributyltin (TBT) and triphenyltin (TPT) at concentrations above $3 \times 10^{-7}$ M, due to cytotoxicity. In JAR cells, $^{35}$S-methionine uptake was decreased by 50% at $3 \times 10^{-7}$ M of TBT. At a TPT concentration of $1 \times 10^{-7}$ M, protein synthesis in JAR cells was not affected, whereas JEG-3 and BeWo cells demonstrated slightly decreases. In all cell lines, both TBT and TPT increased levels of hCG secretion and aromatase activity in a dose- and time-dependent fashion following exposure to nontoxic concentration ranges. In addition, these trialkyltin compounds enhanced 8-bromo-cAMP-induced hCG secretion and aromatase activity in JAR cells. TBT caused dose-related increases in steady-state mRNA levels of both hCGβ and CYP19 in JAR cells following 24- or 48-h exposure to nontoxic concentrations of TBT. However, these mRNA changes in JAR cells were not comparable to the changes in both hCG secretion and aromatase activity. These results indicate that the observed trialkyltin-induced alterations in human choriocarcinoma cells are due to other mechanism in addition to a regulation of hCG and *CYP19* mRNA levels. Our studies suggest that trialkyltin compounds are potent stimulators of human placental hCG production and aromatase activity *in vitro*; and the placenta represents a potential target organ for trialkyltin compounds, whose endocrine-disrupting effects might be the result of local changes in hCG and estrogen concentrations in pregnant women. (*J Clin Endocrinol Metab* 87: 2830–2837, 2002)**

THE PLACENTA PLAYS a vital role in maintaining pregnancy by delivering oxygen and nutrients from the maternal circulation to the fetus and by returning fetal metabolites to the mother. In addition, the placenta performs many crucial endocrine functions. For example, one of the endocrine functions of the human placenta is production of estrogenic steroids (1). The requires the catalytic activity of an aromatase enzyme complex, converting androgenic to estrogenic steroids. This enzyme complex consists of the microsomal CYP19 enzyme and flavoprotein nicotinamide adenine dinucleotide phosphate reduced form-reductase (2, 3). The latter is responsible for transferring reducing equivalents to CYP19 within the membrane of the endoplasmatic reticulum. The human placenta exhibits a high level of aromatase activity and therefore regulates the balance of estrogens *in utero*. The alterations of aromatase function *in utero* has been shown to permanently affect human embryos (4) and increase the risk of both breast and endometrial cancers (5). The human placenta also produces protein hormones, such as human CG (hCG), in addition to steroid hormones. The production and release of hCG is precisely regulated by a number of regulating agents, such as GnRH (6), gonadal steroids (7), epidermal growth factor (8), and cytokines such as IL-1 (9) and IL-6 (10). Maternal serum hCG levels peak between 12 and 13 wk of gestation and then decline to relatively low levels for the remainder of pregnancy. Stimulation from hCG not only governs progesterone production in the corpus luteum during the first trimester (11), but also T production within the fetal testes (12). Given the pivotal functional roles of CYP19 and hCG in sexual development and reproduction, the developmental and reproductive toxicity of environmental contaminants known to have endocrine-disrupting effects might plausibly involve these either or both CYP19 and hCG.

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT) have been widely used as biocides, agriculture fungicides, wood preservatives, disinfecting agents in circulating industrial cooling waters, and as antifouling paints for marine vessels (13, 14). Human exposure to these organotin compounds may result from the consumption of organotin-contaminated meat and fish products, occupational exposure during the manufacture and formulation of organotin compounds, or the application and removal of organotin-containing paints (15, 16). Potential exposure of humans to organotins has therefore aroused great concern about potential toxicities. Most of the toxic effects of organotin compounds on sexual development and reproductive

Abbreviations: 8-Br-cAMP, 8-Bromo-cAMP; CYP19, aromatase; DMSO, dimethylsulfoxide; hCG, human CG; TBT, tributyltin: TPT, triphenyltin.

function have been documented in mollusks. For example, female neogastropod snails have been observed to suffer irreversible sex organ alterations, a phenomenon known as "imposex" (17). These abnormalities are the result of a masculinization process, in which male sex organs develop, notably a penis and a vas deferens. In certain species, growth of a vas deferens disrupts the structure and function of the oviducts, preventing normal breeding activity and causing population decline. Imposex has now been established as a form of endocrine disruption caused by elevated T titers leading to masculinization in organotin-exposed females (18–20). The precise mechanism by which T levels are increased has not been fully elucidated, but the weight of evidence suggests that organotin compounds act as competitive inhibitors of aromatase activity (19). Some recent data suggests that organotin compounds may also inhibit the formation of sulfur conjugates of T and active T metabolites (20). However, in humans, no studies have examined disturbances in sexual development and reproductive function, including placental function, as a result of exposure to organotin compounds.

To be able to apply current knowledge regarding the toxicity of trialkyltin compounds on sexual development and reproduction to humans, the present study assessed the possible effect of trialkyltin compounds on placental aromatase activity and hCG secretion using three different human choriocarcinoma cell lines, JAR, JEG-3, and BeWo. The potential toxicity of trialkyltin compounds as endocrine disruptors in humans is discussed.

## Materials and Methods

### *Chemicals and cell culture*

Tri-n-butyltin chloride (TBT) and tripheniyltin hydrooxide (TPT) were obtained from Tokyo Kasei Kougyo (Tokyo, Japan). 8-bromo cAMP (8-Br-cAMP) was obtained from Nacalai Tesque (Kyoto, Japan). Human choriocarcinoma cell lines JAR, JEG-3, and BeWo, were obtained from ATCC (Manassas, VA). JAR cells (ATCC no. HTB-144) were cultured in Roswell Park Memorial Institute 1640 medium with 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, and 4.5 g/liter glucose. JEG-3 cells (ATCC no. HTB-36) were cultured in MEM with 10% FCS, 2 mM L-glutamine, and a 0.1 mM MEM nonessential amino acid solution. BeWo cells (ATCC no. CCL-98) were cultured in DMEM with 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, and a 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA). All lines were maintained in a humidified atmosphere containing 5% $CO_2$ at 37 C. Cells were grown to confluence and medium was changed every 2–3 d. Confluent cells were subcultured after trypsinization. Cells were treated with either a trialkyltin compound in 0.1% dimethylsulfoxide (DMSO) or vehicle alone (0.1% DMSO). In control experiments, this concentration of DMSO was not found to alter $^{35}S$-labeled methinine incorporation, hCG secretion, or aromatase activity, in any of the cell lines examined.

### *Protein synthesis assay*

To determine cytotoxicity of trialkyltin compounds, $1 \times 10^3$ cells/well were cultured in microtiter plates. After 24 h of culture, cells were treated with various concentrations of trialkyltin compounds. Each culture was pulsed with 50 kilobecquerels of $^{35}S$-labeled methionine (Amersham Pharmacia Biotech, Little Chalfort, Buckinghamshire, UK) for 4 h before cells were harvested. $^{35}S$-count incorporated into TCA-precipitable materials was determined.

### *Determination of hCG in culture media*

Forty-eight well plates were seeded with $4 \times 10^4$ cells/well. After 24 h of culture, cells were treated with various concentrations of trialkyltin compounds in the absence or presence of 8-Br-cAMP for an additional various hours. At the end point of each treatment, cells were washed three times and cultured in fresh medium for another 24 h. Culture supernatant was collected, and hCG was determined using ELISA. Immobilized rabbit polyclonal antibody against intact hCG and primary mouse monoclonal antibody against the β-subunit of hCG conjugated with rat horseradish peroxidase-labeled polyclonal antibody against mouse IgG1 (Zymed Laboratories, Inc., San Francisco, CA) were used for this technique.

### *Aromatase assay*

Twelve-well plates were used to culture $1.5 \times 10^5$ cells/well. After 24 h of culture, cells were treated with various concentrations of trialkyltin compounds in the absence or presence of 8-Br-cAMP for an additional number of hours. At the end point of each treatment, cells were washed three times. Aromatase activity was then determined using the tritium release assay described by Lephart and Simpson (21) with some modifications. This method measures production of $^3H_2O$, which forms as a result of aromatization of substrate [1β-$^3$H] androst-4-ene-3,17-dione (NEN Life Science Products, Boston MA, ). Serum-free medium containing [1β-$^3$H] androst-4-ene-3,17-dione solution (54 nM) was prepared and 0.5 ml of this solution was added to each well. Wells containing media alone were also tested to provide control values for aromatase activity. After incubation for 1 h at 37 C in an atmosphere of 5% $CO_2$, each plate was placed on ice and 200 μl of culture medium was withdrawn from each well. The medium was extracted with 500 μl of chloroform, vortexed, then centrifuged for 1 min at $9,000 \times g$. A 100-μl aliquot of the aqueous phase was mixed with 100 μl of a 5% wt/vol charcoal 0.5% wt/vol dextran T-70 suspension, vortexed, then incubated for 10 min. After centrifugation for 5 min at $9,000 \times g$, a 150-μl aliquot was removed to measure the level of radioactivity.

### *Determination of E2*

Twelve-well plates were used to culture $1.5 \times 10^5$ cells/well. After 24 h of culture, cells were treated with various concentrations of trialkyltin compounds for a further 48 h. At the end point of each treatment, cells were washed three times, and 1 ml of culture medium supplemented with 2 μg androstenedione/ml was added. After incubation for 4 h at 37 C in an atmosphere of 5% $CO_2$, cells were rapidly frozen and thawed, and the total E2 content of the cells and their media was determined by assay with a Correlate-EIA E2 Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI).

### *Determination of intracellular cAMP*

Ninety-six well plates were used to culture $5 \times 10^4$ cells/well. After 24 h of culture, cells were treated with various concentrations of trialkyltin compounds for additional various hours. At the end point of each treatment, cells were washed and the level of intracellular cAMP was determined by assay with a cAMP Enzyme Immunoassay kit (Amersham Pharmacia Biotech, Little Chalfort, Buckinghamshire, UK).

### *Quantitative RT-PCR*

Five micrograms of total RNAs extracted from JAR cells were reverse transcribed using SuperScript II reagent (Invitrogen) and oligo-(deoxythymidine) as primer. The reaction was performed by incubation for 1 h at 50 C in 20 μl of volumes. After termination of cDNA synthesis, reaction mixtures were diluted to 1/10 with TE buffer. Aliquots (2 μl) of diluted reverse transcribed products were amplified in a reaction mixture containing 1× LightCycler-FastStart DNA Master SYBER Green I (Roche Diagnostics, Mannheim, Germany), 4 mM $MgCl_2$, and 0.5 μM of each primer using LightCycler (Roche Diagnostics). After preincubation at 95 C for 10 min, PCR was performed with 35–40 cycles of denaturation at 95 C for 1 sec, annealing at 65 C for 5 sec, and elongation at 72 C for 5 sec. Primers used were as follows: human aromatase, 5′-ATGAATCGGGCCTATGTGGACGTG-3′ and 5′-CGATAGCACTTTCGTCCAAAGGG-3′, hCGβ, 5′-CCGTGTGCATCACCGTCAACA-3′ and 5′-GTTGCACACCACCTGAGGCGA-3′, and human β-actin, 5′-CTACGAGCTGCCTGACGG-3′ and 5′-GCCACAGGACTCCATGCCC-3′.

*Statistics*

Data were analyzed using the two-tailed unpaired *t* test. Control and treatment group data were always obtained from an equal number of replicate experiments. Values of $P < 0.05$ were considered statistically significant.

## Results

*Effects of TBT and TPT on proliferation of human choriocarcinoma cells*

Protein synthesis assays were conducted to determine whether treatment with TBT and TPT was associated with cytotoxic effects on cell division. Cells were treated with various concentrations of trialkyltin compounds for 48 h, and protein synthesis was evaluated by $^{35}$S-labeled methionine incorporation. Exposure to $10^{-8}$ to $10^{-7}$ M of TBT or $10^{-8}$ to $3 \times 10^{-8}$ M of TPT had no significant effect on the $^{35}$S-methionine incorporation of JEG-3 cells and BeWo cells. In JAR cells, protein synthesis was slightly increased at these concentration range of these trialkyltin compounds. At a TBT concentration of $3 \times 10^{-7}$ M, $^{35}$S-methionine incorporation in JAR cells was decreased by 50%, whereas that in JEG-3 cells and BeWo cells was decreased by more than 15%. At a TPT concentration of $10^{-7}$ M, protein synthesis in JAR cells was unaffected, compared with decreases by 70% in JEG-3 cells and 80% in BeWo cells. Moreover, exposure to greater than $10^{-6}$ M TBT or $3 \times 10^{-7}$ M TPT strikingly decreased $^{35}$S-methionine incorporation in all cell lines by more than 5%. Similar results were obtained in parallel experiments, in which cells were treated with trialkyltin compounds for 24 h or 72 h (data not shown). Using this result as a guide, it was considered that exposure of JAR cells to concentrations less than $3 \times 10^{-7}$ M TBT or $10^{-7}$ M TPT, and exposure of JEG-3 cells and BeWo cells to concentrations less than $10^{-7}$ M TBT or TPT would be suitable for investigating the possible effects of these trialkyltin compounds on hCG production and aromatase activity of each cell line.

*TBT and TPT stimulate hCG production in human placental choriocarcinoma cells*

To investigate the possible effects of trialkyltin compounds TBT and TPT on hCG production, JAR, JEG-3, and BeWo cells were incubated with or without TBT or TPT for 48 h. Both TBT and TPT induced dose-related increases in hCG secretion by all choriocarcinoma cells (Fig. 2). Amounts of hCG released increased significantly as TBT concentration increased from $10^{-8}$ to $3 \times 10^{-7}$ M or TPT concentrations increased from $10^{-8}$ to $10^{-7}$ M (Fig. 2). Above these concentration ranges, hCG release declined because of cytotoxicity. This is represented by the degree of $^{35}$S-methinine incorporation (Fig. 1). The observed induction of hCG secretion demonstrated a strong tendency following treatment with TPT rather than TBT (not significant). The hCG secretion induced by both compounds was significantly greater than positive controls induced by RA, which is known to stimulate the secretion of hCG in placental choriocarcinoma cells (22).

*TBT and TPT enhance aromatase activity in human placental choriocarcinoma cells*

We then investigated the possible effect of TBT and TPT on aromatase activity in JAR, JEG-3, and BeWo cells. Some investigators have demonstrated that various agents can effect aromatase expression and activity in choriocarcinoma cells when maintained in serum-free medium (21, 23, 24). Serum-free medium was used because serum has been shown to inhibit the inductive effects of several agents on aromatase expression and activity in several cell types (21, 24). However, studies on phorbol ester stimulation of aromatase expression and activity in choriocarcinoma cells, as well as studies examining cAMP stimulation in human term trophoblast cells, have been performed in the presence of serum (25). Regardless, to acquire more precise information on the toxic effects of xenobiotics through *in vitro* study, we suggest that future experiments should be performed in the presence of serum to reflect *in vivo* conditions. Thus, in the present study, choriocarcinoma cells were incubated with or without TBT or TPT for 48 h, in the presence of serum. Both TBT and TPT caused a dose-dependent increase in the aromatase activity of all choriocarcinoma cells at the concentration up to $10^{-7}$ M (Fig. 3). Exposure to greater than $10^{-8}$ M TBT or $3 \times 10^{-8}$ M TPT caused statistically significant increases in aromatase activity in all choriocarcinoma cell lines examined. Notably, JAR cells exhibited the greatest sensitivity to TBT in aromatase activity, with statistically significant increases ($P < 0.005$) in aromatase activity in JAR cells observed after treatment with only $3 \times 10^{-9}$ M TBT (Fig. 3). Although a statistically significant increase in aromatase activity was observed in all choriocarcinoma cell lines after exposure to $10^{-8}$ M TBT, this was not observed in JAR cells and BeWo cells after treatment with $10^{-8}$ M TPT (Fig. 3).

In addition, we measured E2 accumulation within JAR cells and within culture medium after treatment with TBT

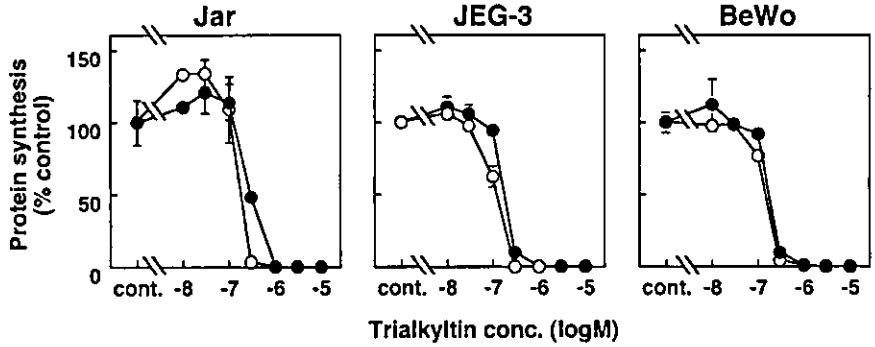


FIG. 1. Cytotoxicity of trialkyltin compounds against human choriocarcinoma cell lines. Cells treated with varying concentrations of either TBT (●) or TPT (○) for 48 h. Results expressed as mean ± SD of triplicate cultures.

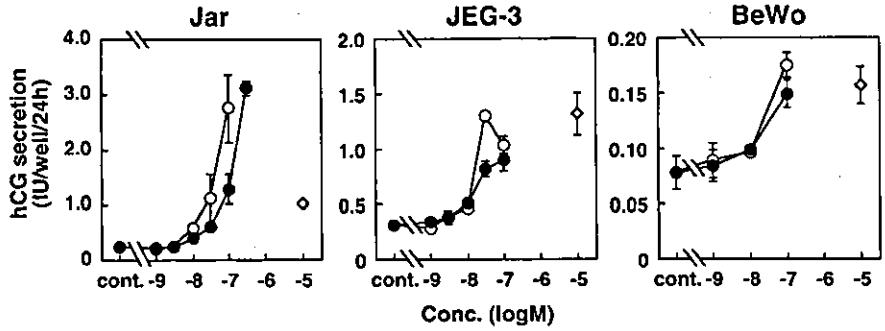


FIG. 2. Effect of trialkyltin compounds on hCG secretion in human choriocarcinoma cell lines. Cells treated with varying concentrations of TBT (●), TPT (○), or RA (◇), for 48 h. Results expressed as mean ± SD of triplicate cultures.

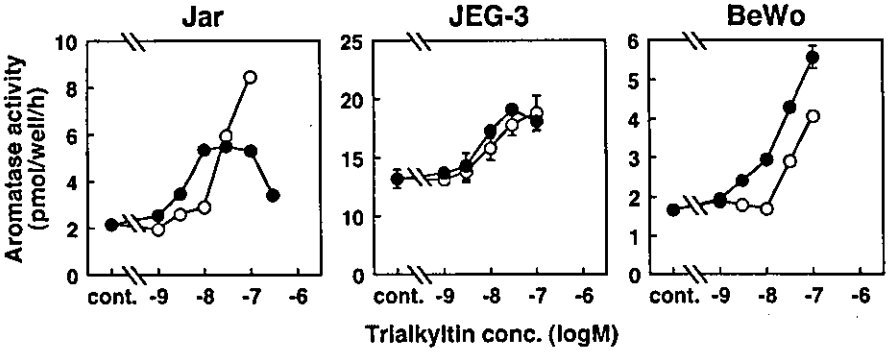


FIG. 3. Effect of trialkyltin compounds on aromatase activity in human choriocarcinoma cell lines. Cells treated with varying concentrations of TBT (●) and TPT (○) for 48 h. Results expressed as mean ± SD of triplicate cultures.

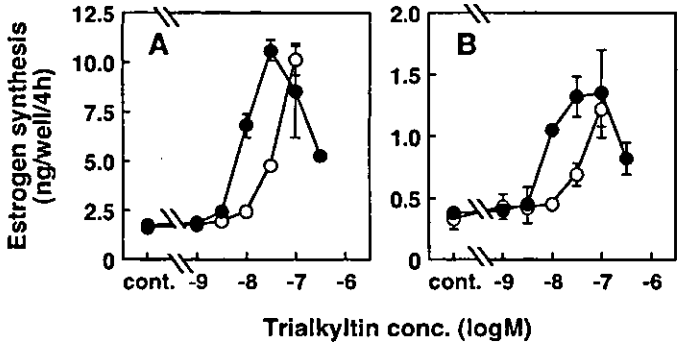


FIG. 4. Effect of trialkyltin compounds on conversion of androstenedione to E2 by JAR cells. Cells treated with varying concentrations of TBT (●) and TPT (○) for 48 h. E2 content of cell media (A), and cells (B), determined by ELISA. Results expressed as mean ± SD of triplicate cultures.

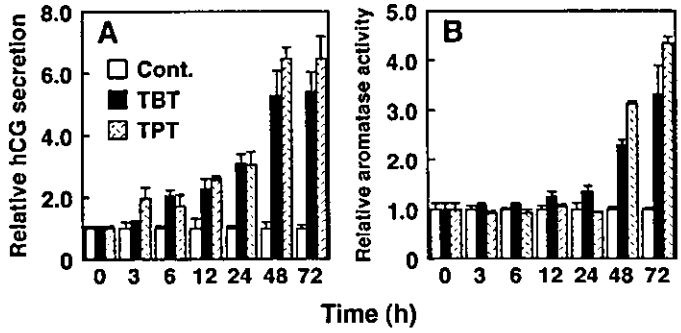


FIG. 5. Time-course study of effects of trialkyltin compounds on hCG secretion (A) and aromatase activity (B) in JAR cells. JAR cells treated with concentrations of $10^{-7}$ M TBT (●) and TPT (○) for indicated periods. Results expressed as mean ± SD of triplicate cultures.

and TPT. Parallel to the observed increase in aromatase activity, E2 accumulation in JAR cells significantly increased upon cultivation with TBT and TPT (Fig. 4). Concentrations under $10^{-6}$ M of either trialkyltin compound did not significantly affect aromatase activity in microsomes isolated from JAR cells (data not shown). These results suggest that the trialkyltin compounds tested (TBT and TPT) did not exert effects on the enzyme complex at concentrations below $10^{-6}$ M.

*Time course of the effects of trialkyltin compounds on hCG secretion and aromatase activity in JAR cells*

Both the level of hCG production and aromatase activity in JAR cells were increased in a time-dependent manner of treatment with $10^{-7}$ M of TBT or TPT (Fig. 5). The level of hCG production increased significantly after 3 h and 6 h treatment with TPT and TBT, respectively (3 h TPT; 197 ± 36%, 6 h TBT; 205 ± 18%, of control secretion). Conversely, levels of aromatase activity increased slightly but significantly (135 ± 12% of control activity) after 12 h treatment with TBT. Furthermore, 48 h exposure was necessary to achieve significant changes in aromatase activity induced by TPT at least (Fig. 5).

*Effects of trialkyltin compounds on intracellular cAMP accumulation, and 8-Br-cAMP induced-hCG secretion and aromatase activity in JAR cells*

Both hCG production and aromatase activity in the placenta are controlled by cAMP-dependent intracellular signal pathways. To determine whether hCG release and aromatase activity mediated by trialkyltin compounds was related to increased intracellular cAMP, we measured cAMP concentrations in JAR cells after 24 h or 48 h treatment with TBT and TPT. Neither trialkyltin compounds demonstrated any effect on cAMP production in JAR cells at all concentrations tested (Fig. 6), suggesting that hCG production and aromatase ac-

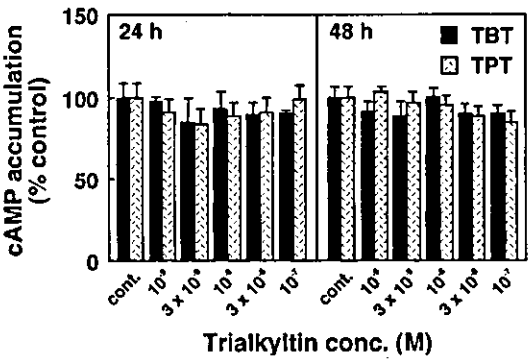


FIG. 6. Effect of trialkyltin compounds on cAMP production in JAR cells. JAR cells cultured with varying concentrations of TBT or TPT for indicated periods. Results expressed as mean ± SD of triplicate cultures. Intracellular cAMP levels for control incubates were 58.2 ± 2.5 fmol/well at 24 h, 84.6 ± 2.9 fmol/well at 48 h.

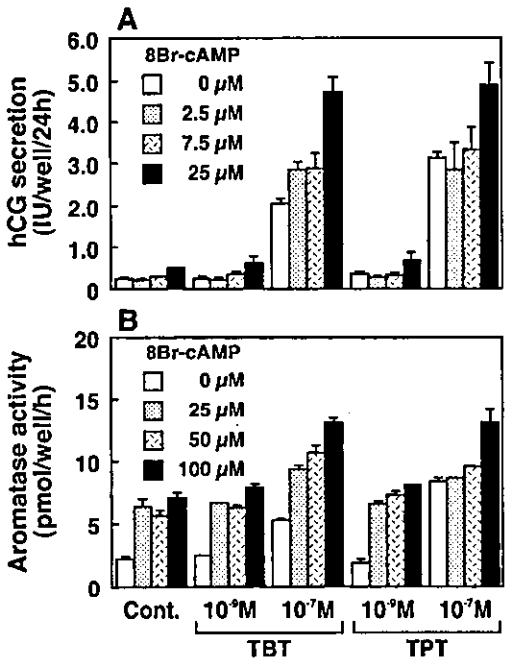


FIG. 7. Effect of trialkyltin compounds on 8-Br-cAMP induced hCG production and aromatase activity in JAR cells. JAR cells stimulated with varying concentrations of 8-Br-cAMP in absence or presence of varying concentrations of TBT or TPT for 48 h. Results expressed as mean ± SD of triplicate cultures.

tivity is induced by the trialkyltin compounds through a cAMP-independent pathway.

We then examined the effects of these trialkyltin compounds on 8-Br-cAMP (a cAMP homolog) stimulated-hCG production and aromatase activity in JAR cells. As shown in Fig. 7A, TBT and TPT combined exhibited synergistic effects on hCG release with 8-Br-cAMP over the examined concentration ranges. TBT exhibited additive effects on aromatase activity with 8-Br-cAMP over the examined concentration range (Fig. 7B). However, TPT exhibited additive effects on aromatase activity with 8-Br-cAMP at 100 μM but not at concentrations below 50 μM (Fig. 7B). These results clearly indicate that both TBT and TPT enhance hCG secretion and aromatase activity stimulated by intracellular signals via cAMP-dependent pathway.

*Effects of TBT on mRNA expression of hCGβ and CYP19 in JAR cells*

We then investigated TBT-induced hCG and aromatase (*CYP19*) mRNA expression in JAR cells by quantitative real-time RT-PCR (Fig. 8). The mRNA level of hCGβ increased slightly but not significantly as a result of 24 h treatment of JAR cells with TBT. Exposure to $3 \times 10^{-8}$ to $3 \times 10^{-7}$ M of TBT induced a significant increase after 48 h treatment at last. In JAR cell, however, hCG production was markedly increased by 48 h treatment with TBT ($3 \times 10^{-8}$ M; 243 ± 6%, $10^{-7}$ M; 518 ± 109%, $3 \times 10^{-7}$ M; 1250 ± 53% of control expression; Fig. 2), whereas the mRNA expression of hCGβ was barely increased by 48 h treatment with TBT ($3 \times 10^{-8}$ M; 148 ± 7%, $10^{-7}$ M; 212 ± 42%, $3 \times 10^{-7}$ M; 163 ± 19% of control expression; Fig. 8A).

The mRNA level of *CYP19* also increased slightly but significantly as a result of 24 h treatment of JAR cells with TBT at concentrations of $10^{-7}$ M (150 ± 1% of control expression) and $3 \times 10^{-7}$ M (240 ± 35% of control expression). After 48 h treatment with TBT, values increased to 187 ± 26%, 216 ± 18% and 429 ± 111% of control expression at concentrations of $3 \times 10^{-8}$, $10^{-7}$ and $3 \times 10^{-7}$ M, respectively (Fig. 8B). Although aromatase activity was significantly increased after 48 h treatment with $3 \times 10^{-9}$ M TBT (Fig. 3), mRNA expression of *CYP19* was not increased (Fig. 8B). Neither hCGβ nor *CYP19* demonstrated increased mRNA expression following treatment with TBT for less than 24 h (data not shown). These data suggest that hCG production and aro-

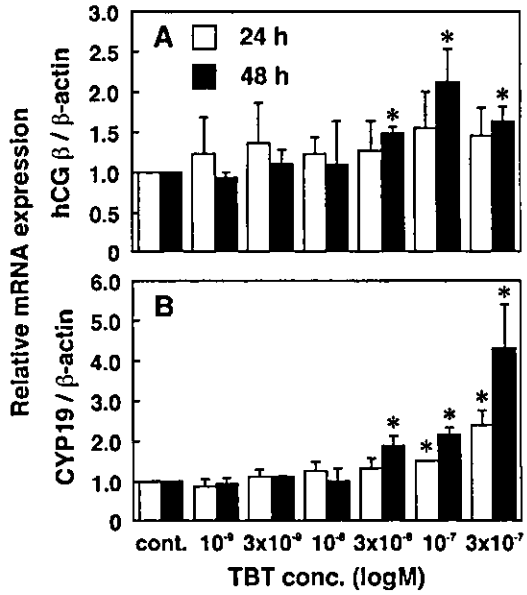


FIG. 8. Effect of TBT on expression of hCG β and CYP19 mRNA in JAR cells. Total RNA isolated from JAR cells treated with varying concentrations of TBT for indicated time periods. Relative hCG β and CYP19 mRNA levels for each condition determined by quantitative RT-PCR three times for each of three independent cultures as described in *Materials and Methods*. Results expressed as mean ± SD of three independent cultures. *, $P < 0.05$ represent values significantly different controls.

matase activity induced by treatment with TBT are due to other mechanisms in addition to increases in mRNA expression.

## Discussion

The results of the experiments described in this study show that trialkyltin compounds enhance hCG secretion and aromatase activity in human choriocarcinoma cell lines when they are exposed to nontoxic concentrations. The observed induction of hCG secretion demonstrated a strong tendency toward treatment with TPT rather than TBT, whereas induction of aromatase activity exhibited the reverse trend. In addition, we demonstrated that trialkyltin compounds cause dose- and time-dependent increases in hCG and *CYP19* mRNA levels in human choriocarcinoma cells. However, no effects were exerted on intracellular cAMP production, which is involved in an intracellular signals pathway to promote hCG and *CYP19* mRNA expression. Furthermore, mRNA changes in JAR cells treated with TBT were not comparable to changes in both hCG secretion and aromatase activity. Consequently, we concluded that the observed trialkyltin-induced alterations in human choriocarcinoma cells are due to other mechanisms in addition to regulation of hCG and *CYP19* mRNA levels.

Previously, hCG has been shown to stimulate placental aromatization during *in situ* perfusion of the placenta (26). In addition, Ackermann *et al.* (27) have demonstrated that hCG secretion increases when JEG-3 cells are cultivated under serum-free conditions and that the amount of hCG secreted parallels increases in microsomal aromatase activity. Thus, they have suggested that placental aromatase is induced by hCG in an autocrine fashion. As a result of these reports, we expected that increased levels of hCG might enhance aromatase activity in human choriocarcinoma cells induced by trialkyltin compounds. However, when hCG was added to the media of three human choriocarcinoma cell lines, aromatase activity was not affected (data not shown). Indeed, Ackermann *et al.* (27) did not establish whether hCG could stimulate aromatase activity in JEG-3 cells. Moreover, hCG binds to surface receptors on cells and transmits its message intracellularly via cAMP (28). Nevertheless, intracellular cAMP accumulation is not found in JAR cells following exposure to trialkyltin compounds (Fig. 6). Although it remains unclear whether human placental aromatase is induced by hCG, it appears that hCG is not involved in the induction of placental aromatase by trialkyltin compounds.

Recently, organotin compounds have become recognized as endocrine-disrupting chemicals because numerous marine organisms have been shown to exhibit sexual abnormalities following exposure to TBT and TPT. In gastropod mollusks, one of the most sensitive species to organotin compounds, TBT has been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, a phenomenon known as imposex (17). Although it has been theorized that TBT increases androgen levels through inhibition of aromatase activity and/or a suppression of androgen excretion (18–20), no clear evidence has been provided to verify this hypothesis. On the contrary, a recent report has demonstrated that aromatase activity is not decreased in gastropods exposed to TBT, even in those exhibiting clear evidence of imposex (29). Although Heidrich *et al.* (30) demonstrated that TBT inhibited human aromatase activity at concentrations above $10^{-6}$ M using human placental microsomes and NADPH experimental system, Cooke (31) reported that TBT did not inhibit aromatase activity at concentrations under $10^{-6}$ M. Furthermore, trialkyltin compounds demonstrated no direct effects on the aromatase enzyme complex of JAR cells at concentrations below $10^{-6}$ M in the present study. Exactly why the actions of trialkyltin compounds with regard to aromatase activity differ in humans and gastropods is uncertain. It may be due to large variations in the cytochrome P450 enzymes of the two species. However, our findings suggest that the endocrine disrupting action of trialkyltin compounds observed in the present study might involve a mechanism unique to humans.

Many of the endocrine alterations induced by environmental contaminants are thought to occur as a result of altered gene expression, mediated by the binding of environmental contaminants to steroid hormone receptors. If TBT and TPT are capable of activating general steroid hormone receptors, they might induce ER- or AR-mediated gene expression. However, several studies have reported that neither TBT nor TPT exhibit estrogenic activity in a yeast two-hybrid screening system (32) or a MCF-7 human breast cancer cell line (33). In the present study, hCG secretion and aromatase activity in human choriocarcinoma cell lines were not affected by treatment with E2 (data not shown). On the other hand, Himeno *et al.* (33) have reported induction of AR-mediated transcription by trialkyltin compounds in the LNCaP cell line, an AR human prostate cancer cell line. However, in the present study, hCG secretion and aromatase activity in human choriocarcinoma cells were not affected by treatment with T (data not shown). This suggests that steroid hormone receptors do not mediate trialkyltin-induced hCG secretion and aromatase activity in human choriocarcinoma cells.

Several reports have established the *in vivo* reproductive toxicity of trialkyltin compounds in rodents (34–39). However, the teratogenicity of trialkyltin compounds remains unclear because it is difficult to evaluate whether they exert direct action on the fetus or whether embryotoxicity results from maternal toxicity. The present study has demonstrated that trialkyltin compounds alter endocrine function in placental cells *in vitro*, but whether the endocrine disrupting effects of trialkyltin compounds could (partly) be caused by local changes in estrogen and hCG concentrations of the placenta *in vivo* remains unclear. The *in vivo* endocrine effects of environmental contaminants on the human placenta from animal studies are difficult to estimate, particularly those involving rodents, because the endocrine functions of the placenta vary considerably among different species.

Aromatization and the secretion of glycoprotein hormones are very important placental endocrine functions during pregnancy in humans. In primates and various ungulate species such as cows, pigs, and horses, aromatase is expressed in the placenta, in addition to other gonadal tissues (40–43). By contrast, the placentae of fetal rats and mice do not express aromatase (44, 45). However, glycoprotein hor-

mones, including hCG, are structurally similar between the two species, each consisting of a common α-subunit and a unique, noncovalently associated β-subunit. A gene encoding the α-subunit of glycoprotein hormones is also expressed in the placentae of primates and various ungulate species, but not in rodents (46, 47). It has been suggested that rodents are therefore unsuitable for evaluating the endocrine-disrupting effects of environmental contaminants in the human placenta.

In addition, the tissue-specific expression of aromatase is strictly regulated in humans. Human *CYP19* is a single-copy gene composed of 10 exons; exons II-X encode for the aromatase protein, as well as the 3′ untranslated region of mRNA common to all estrogen-producing tissues (48). A number of variations of exon I exist. These encode for the 5′ untranslated regions of various *CYP19* mRNAs, which are selectively expressed in some tissues by alternative splicing (48). The tissue-specific expression of *CYP19* in humans appears to be mediated by tissue-specific promoters lying upstream of the respective exon I sequences, and by transcription factors binding to specific regions of each promoter. In placenta, CYP19 is driven by the placental major promoter (I.1) and the transcript contains exon I.1, located on approximately 89-kb upstream from exon II (49). On the other hand, ovarian transcripts contain a sequence at 5′-ends immediately upstream of the translation start site, because expression of the gene in the ovary uses a proximal promoter (II) (49). Recently, Saitoh *et al.* (50) reported that TBT inhibits aromatase activity and decreases mRNA level in ovarian granulosa cells. In these cells, expression of CYP19 is strongly regulated by steroidogenic tissue-specific transcriptional factor, Ad4Bp/SF-1, via promoter II. In contrast, Ad4Bp/SF-1 is expressed at very low levels in human placenta and may not play an important role in activation of placental major promoter I.1 (51, 52). This suggests that the effects of TBT in these cells were partly caused by association with Ad4Bp/SF-1. It is therefore likely that the action of TBT in JAR cells is induced by a pathway clearly differ from that in ovarian granulosa cells, and giving rise to the promotion of aromatase activity and mRNA expression. From these opposite effects of TBT for aromatase activity and expression in different tissue-derived cells, the regulation of tissue-specific aromatase expression would appear to be strictly controlled.

Results of studies using rapid amplification of cDNA ends cDNA libraries constructed from cattle, horse, and pig placental tissues have indicated that tissue-specific promoters lying upstream of placenta-specific exons also regulate placental expression of *CYP19* genes in these species (40). In species in which the 5′ flanking regions of *CYP19* placenta-specific promoters have been cloned, low sequence identity is seen among species (40, 41). Furthermore, in humans, the placenta-specific promoter of the *CYP19* gene lies approximately 90 kb upstream from the site of initiation of translation (49), whereas in bovines, the placenta-specific promoter of the *CYP19* gene lies approximately 19 kb upstream of the site of initiation of translation (42). In the pig, even greater complexity is observed because as many as three *CYP19* genes encode for tissue-specific isoforms and multiple promoters can initiate translation of some of these isoforms (41, 43). Hence, any evaluation of the endocrine-disrupting effects of environmental contaminants on placental aromatase should account for this complexity.

To our knowledge, this is the first study investigating an interaction between trialkyltin compounds and human placental endocrine functions. Ongoing studies in our laboratory are currently investigating other cell lines expressing both aromatase and hCG. Furthermore, we plan to identify the regulatory region involved in trialkyltin-induced hCG and *CYP19* expression in the placenta. These studies will provide useful information for assessing human risk in exposure to trialkyltin compounds *in utero*.

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