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Assessing Hormone Receptor Activities of Pyrethroid Insecticides and Their Metabolites in Reporter Gene Assays

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Pyrethroid insecticides, the most commonly used insecticides worldwide, are suspected endocrine-disrupting chemicals. But their interactions with hormone receptors are still unclear. The present study intended to evaluate and compare the hormone receptor (estrogen receptor [ER], androgen receptor [AR], and thyroid hormone receptor [TR]) activities of nine pyrethroids (cycloprothrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, etofenprox, fenvalerate, permethrin, and tetramethrin) and their metabolites (3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropne carboxylic acid [DCCA] and 3-phenoxybenzoic acid [3-PBA]) using receptor-mediated luciferase reporter gene assays. Of the 11 compounds tested, four showed very weak ER agonistic activities and six displayed antiestrogenic effects, among which cyhalothrin and DCCA possessed the most potent estrogenic and antiestrogenic activity respectively. Antagonistic effects to AR were found in 7 compounds, with cyfluthrin and deltamethrin exhibiting stronger AR antagonistic capacity. In the TR assay, all of tested chemicals except DCCA showed antagonistic effects. In this study, we provided evidence that a variety of pyrethroids and their metabolites might disrupt the function of multiple nuclear hormone receptors and thus have the potentials to affect the endocrine and the reproductive systems in humans.

Key Words: **pyrethroid insecticides; ER; AR; TR; reporter gene assay.**

Substances with endocrine activities in humans and wildlife are known as endocrine-disrupting chemicals (EDCs). Their potential effects constitute a major concern among the public and the scientific community (Colborn *et al*., 1993; Diamanti-Kandarakis *et al*., 2009). The major mechanism by which EDCs exert their potential disruptive effects on physiological process is through interaction with nuclear hormone receptors. Chemicals mimicking or inhibiting endogenous hormone action via estrogen receptor (ER) and androgen receptor (AR) have been the focus of research in recent years. Some studies also target thyroid hormone receptor (TR) (DeVito *et al*., 1999; Sun *et al*., 2009).

Pesticides for crop protection and residential pest control, including pyrethroids, are one big group of EDCs. Risk mitigation decisions made by the U.S. Environmental Protection Agency (EPA) have resulted in an increased utilization of pyrethroid insecticides and thus an increase of human exposure to pesticides. Pyrethroids are synthetic chemicals similar to pyrethrins made by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium*) (Casida, 1980). However, they have been modified to increase their insecticidal potency and stability in the environment. Pyrethroids and their metabolites have been identified in various environmental sectors, including soil, aquatic microcosms (Erstfeld, 1999), sediment (Gan *et al*., 2005), and residues in food (Markovic *et al*., 2010). More surprisingly, these compounds have been detected in human samples, such as breast milk (Sereda *et al*., 2009) and urine (Xia *et al*., 2008).

Recently, pyrethroids have been linked to disruption of the endocrine system, which can adversely affect reproduction and sexual development as well as the immune system (Bian *et al*., 2004; Pine *et al*., 2008). Several pyrethroids have been assessed for potential endocrine-disrupting activity by various methods. Some pyrethroids showed estrogenic activities in MCF-7 cell proliferation and pS2 mRNA expression assays (Chen *et al*., 2002; Go *et al*., 1999; Zhao *et al*., 2008), whereas a few pyrethroids were found to have weak antiestrogenic activity (Kojima *et al*., 2004). The antiandrogenic effects of some pyrethroids have received attention (Xu *et al*., 2008; Zhang *et al*., 2008). But some pyrethroids showed no (anti)estrogenic or antiandrogenic activity in other studies (Andrade *et al*., 2002; Saito *et al*., 2000). These results are somewhat contradictory, probably because of the different sensitivity and specificity of *in vitro* and *in vivo* test systems. Meanwhile, only a few reports investigated the effects of pyrethroids on thyroid hormone (TH) level and thyroid gland using *in vivo* assays (Akhtar *et al*., 1996; Maiti and Kar, 1998), and their disruptive effects on the binding of THs to their

receptors are rarely studied. Therefore, further characterizing the endocrine-disrupting effects of pyrethroids, especially their hormone receptor activities, is required for assessing potential health risks.

Transactivation or reporter gene assay has been established as a powerful tool for testing receptor agonists and antagonists among chemicals (USEPA, 2008). This kind of assay is based upon the expression of a reporter gene induced by a chemical through the ligand-receptor binding and subsequent transcriptional activation pathway. We previously modified this assay to achieve sensitivity and specificity to chemicals and provided evidence that a variety of pesticides have both agonistic and antagonistic activities against estrogen receptor α (ERα), AR, and thyroid hormone receptor β (TRβ) (Shen *et al*., 2009; Sun *et al*., 2009).

In the present study, we tested the hormone receptor activities of ERα, AR, and TRβ against nine commercial pyrethroids and two metabolites of pyrethroids using our reporter gene assays. Cycloprothrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, etofenprox, fenvalerate, permethrin, and tetramethrin were chosen according to their wide and frequent of use in China. Two metabolites, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropne carboxylic acid (DCCA) and 3-phenoxybenzoic acid (3-PBA), which are nonspecific metabolites of several different pyrethroid insecticides, were also selected in these assays. These studies are intended to provide novel information about the potential hormone receptor activities of a variety of pyrethroid insecticides and their metabolites.

MATERIALS AND METHODS

Chemicals. Nine commercial pyrethroids and two metabolites of pyrethroids were purchased from Labor Dr Ehrenstorfer-Schäfers (Augsburg, Germany), at the highest purity commercially available. The purity, Chemical Abstract Service, and abbreviation of chemicals were listed in Table 1. 17β-estradiol (E2, ≥ 99%), 5α-dihydrotestosterone (5α-DHT, ≥ 97.5%), and L-3,5,3′-triiodothyronine (T3, ≥ 98%) were purchased from Sigma (St Louis, MO). Chemical structures of the pyrethroid pesticides tested in this study were shown in Figure 1. Stock solutions of the chemicals were prepared in dimethylsulfoxide (DMSO) at a concentration of 10^{-1}M, stored at −20°C, and diluted to desired concentrations in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Sigma) (ER and TR reporter gene assay) or Leibovitz's L-15 medium (Sigma) (AR reporter gene assay) immediately before use. The final concentration of DMSO in the culture medium did not exceed 0.1% (vol/vol) that did not affect cell yields according to our previous experiments (Sun *et al*., 2009).

TABLE 1
Data on Tested Chemicals

Chemicals	Chemical Abstract Service	Purity (%)
Cycloprothrin	63935-38-6	95.5
Cyfluthrin	68359-37-5	98.0
Cyhalothrin	91465-08-6	98.0
Cypermethrin	52315-07-8	92.0
Deltamethrin	52918-63-5	99.0
Etofenprox	80844-07-1	99.0
Fenvalerate	51630-58-1	98.5
Permethrin	52645-53-1	94.0
Tetramethrin	7696-12-0	96.5
3-PBA	3739-38-6	99.0
DCCA	55701-05-8	99.0

Plasmids and cell lines. The luciferase reporter plasmid pERE-TATA-Luc+ including three copies of the *Xenopus laevis* vitellogenin A estrogen-responsive element, rat α_{2u} globulin promoter, and rERα/pCI containing the full open reading frame of rat ERα cDNA were used in the ER reporter gene assay (kindly provided by Dr M. Takeyoshi, Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). Plasmids pGal4-L-TRβ and pUAS-tk-Luc used in the TR reporter gene assay were kindly provided by Dr Ronald M. Evans (Gene Expression Laboratory, Howard Hughes Medical Institute, San Diego, CA). The ligand-binding domain (LBD) of TRβ was fused to the DNA-binding domain (DBD) of Gal4 in plasmid pGal4-L-TRβ. Gal4-responsive luciferase reporter plasmid pUAS-tk-Luc contained four copies of the Gal4-binding site (UAS). The plasmid phRL-tk (used as internal control for transfection efficiency and the cytotoxicity of tested chemicals), containing *Renilla* luciferase gene, was purchased from Promega, Madison, WI. African green monkey kidney cell line CV-1, which did not contain the endogenous receptors (AR, ER, and TR), was obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China. MDA-kb2 cell line was purchased from ATCC, Manassas, VA.

MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to detect the cytotoxicity of the tested chemicals. The CV-1 cells and MDA-kb2 cells attached on culture dishes were collected and planted at a density of 1×10^4 cells/100 μl in each well of 96-well plates in DMEM or L-15 medium with 10% charcoal-dextran–stripped fetal bovine serum (CDS-FBS) (Sigma). Twenty-four hour later, the CV-1 cells were treated with vehicle or various concentrations (10^{-9} to 10^{-4}M) of the test chemicals alone or with 5×10^{-9}M T3 or 1×10^{-9}M E2. The MDA-kb2 cells were exposed to vehicle or various concentrations (10^{-9} to 10^{-4}M) of the test chemicals alone or with 1×10^{-9}M DHT. These cells were incubated at 37°C for 24 h, then 25-μl MTT (5 mg/ml in PBS, Sigma) was added to each well and incubated continuously at 37°C for 4 h. Finally MTT solutions were replaced with 150-μl dimethyl sulfoxide and shaken for 10 min to solubilize the crystals. Absorbance was measured by automatic microplate reader (EL808, Bio-Tek, Winooski, VT) at 570 nm.

ER reporter gene assay. The CV-1 cells were maintained in DEME (Sigma) with 10% FBS (Gibco, Invitrogen Corp., Carlsbad, CA), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in a 37°C, 5% CO_2 incubator. The host cells were plated in 48-well microtiter plates at a density of 0.5×10^5 cells per well in the phenol red-free DMEM medium containing 10% CDS-FBS. Following 12-h incubation, each well was added with 0.5 μg of pERE-TATA-Luc+, 0.2 μg of rERα/pCI, and 0.1 μg of phRL-tk using 2.5-μg Sofast (Sunma Company, Xiamen, China) transfection reagent. After another 12-h incubation, for estrogenic activity tests, cells were treated with various concentrations of tested chemicals, various concentrations of E2 (10^{-11} to 10^{-7}M) (positive control), or 0.1% DMSO (negative control); for antiestrogenic activity, cells were treated with various concentrations of tested chemicals with1×10^{-9}M of E2. DMSO concentrations in the wells never exceed 0.1% (vol/vol). The cells were harvested after 24-h treatment. After rinsed three times with PBS, pH 7.4, the cells were lysed with 1× passive lysis buffer (Promega) and then analyzed immediately using a 96-well plate luminometer (Berthold Detection System, Pforzheim, Germany). The amounts of luciferase and *Renilla* luciferase were measured with the luciferase reporter assay system kit (Promega). The relative transcriptional activity was converted to fold induction above the corresponding vehicle control value (*n* fold). Triplicate samples were run for each treatment.

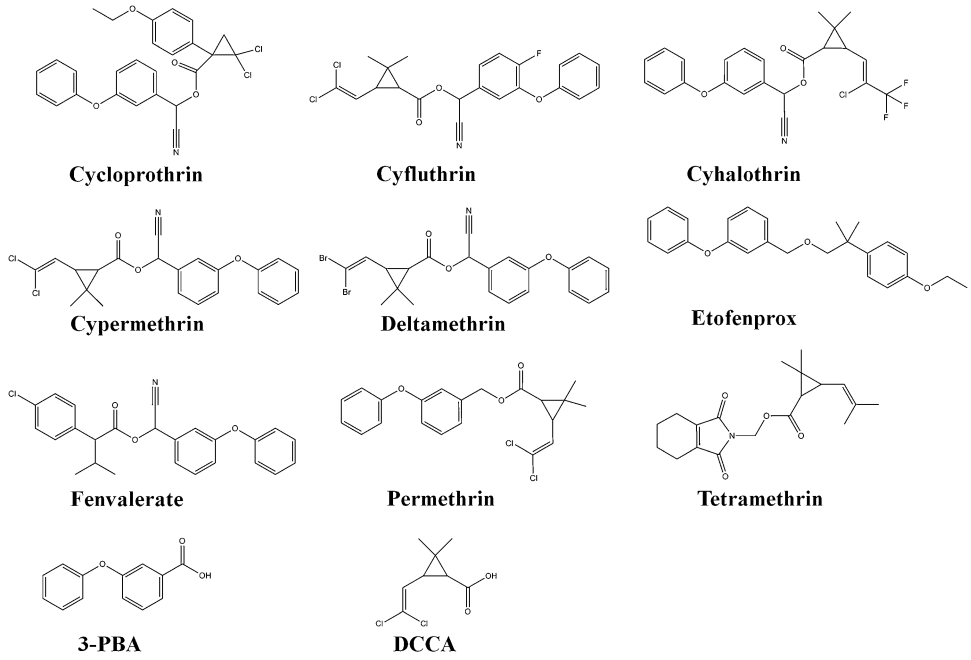

FIG. 1. Chemical structures of pyrethroids and metabolites tested in this study.

AR reporter gene assay. MDA-kb2 cell line had been stably transfected with the pMMTV.neo.luc reporter gene plasmid by Dr Wilson in U.S. EPA. The cells were maintained at 37°C without CO_2 in Leibovitz's L-15 medium supplemented with 10% FBS, 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 0.25 μg/ml amphotericin B (Sigma). MDA-kb2 cells were plated at 1×10^4 cells per well in 100 μl of medium in 96-well microplate. When cells were attached (4–6 h later), they were treated with various concentrations of tested chemicals with or without 1×10^{-9}M DHT, various concentrations of 5α-DHT (10^{-12} to 10^{-7}M) (positive control), or 0.1% DMSO (negative control). Because the MDA-kb2 cell line contains both endogenous AR and glucocorticoid receptor (GR), both of the receptors can bind to and activate the MMTV-luc response element. To distinguish whether agonist activity of tested chemicals was AR or GR mediated, cells were treated with compounds alone and with 1×10^{-6}M flutamide, the potent antagonist of AR. After 24-h incubation, medium was removed from each plate. Plates were washed, and cells were harvested with 25-μl lysis reagent per well. Then the cell lysates were analyzed immediately as described above. The relative transcriptional activity was converted to fold induction above the corresponding vehicle control value (*n* fold). Triplicate samples were run for each treatment.

TR reporter gene assay. The CV-1 cells were cultured and plated as described above, transfected with 0.25-μg pUAS-tk-luc, 0.1-μg pGal4-L-TR, and 0.05-μg phRL-tk using 2.5-μg Sofast transfection reagent in each well. For agonistic activity tests, the CV-1 cells were exposed to various concentrations of tested chemicals, various concentrations of T3 (10^{-11} to 10^{-6}M) (positive control), or 0.1% DMSO (negative control). As for antagonistic activity, the CV-1 cells were treated with various concentrations of tested chemicals with 5×10^{-9}M T3. DMSO concentrations in the wells never exceed 0.1% (vol/vol). Then the CV-1 cells were handled as described in ER reporter gene assay. The relative transcriptional activity was converted to fold induction above the corresponding vehicle control value (*n* fold). Triplicate samples were run for each treatment.

Data analysis. The agonistic activity of tested chemicals was recorded as 20% relative effective concentration (REC_{20}) (Kojima *et al*., 2004), which was defined as the concentration at which the tested chemicals showing 20% of the maximal activity of E2, DHT, and T3. The antagonistic activity of tested chemicals was recorded as 20% relative inhibitory concentration (RIC_{20}) (Kojima *et al*., 2004), which refers to the concentration at which the tested chemicals showing a 20% reduction in the activity of 1×10^{-9}M E2 via ERα, 1×10^{-9}M DHT via AR, and 5×10^{-9}M T3 via TRβ, respectively. REC_{20} and RIC_{20} were calculated by nonlinear regression analysis using Sigmoidal dose-response (variable slope) curve fitting with GraphPad Prism 5.01 for windows (GraphPad Software Inc., San Diego, CA). Data sets were tested for homogeneity of variance and normality first, and all the data sets met these criteria. Then data were analyzed by one-way ANOVA, followed by Duncan's multiple comparisons test when appropriate using SPSS 13.0 (SPSS, Inc.). The level of significance was set at $p < 0.05$. For hormone agonists, treatments were compared with the vehicle control groups; whereas for antagonists, treatments were compared with the 1×10^{-9}M E2, 1×10^{-9}M DHT, and 5×10^{-9}M T3 positive control groups respectively.

RESULTS

Cytotoxicity of the Tested Chemicals

The cytotoxic concentrations of tested chemicals were determined by MTT assay before performing receptor assay. Tested chemicals ($\leq 10^{-5}$M) did not affect the viability and proliferation of CV-1 cell, with or without E2 and T3. They did not affect the viability and proliferation to MDA-kb2 cell, with or without DHT in MTT assay, either. No cytotoxic effects were observed by microscopic examination throughout the transfection assay. Furthermore, the cytotoxicities of the chemicals were assessed by the expression of *Renilla* luciferase produced by the plasmid phRL-tk that was cotransfected to the cells. There was no significant decrease between the tested group and the vehicles control group in the expression of

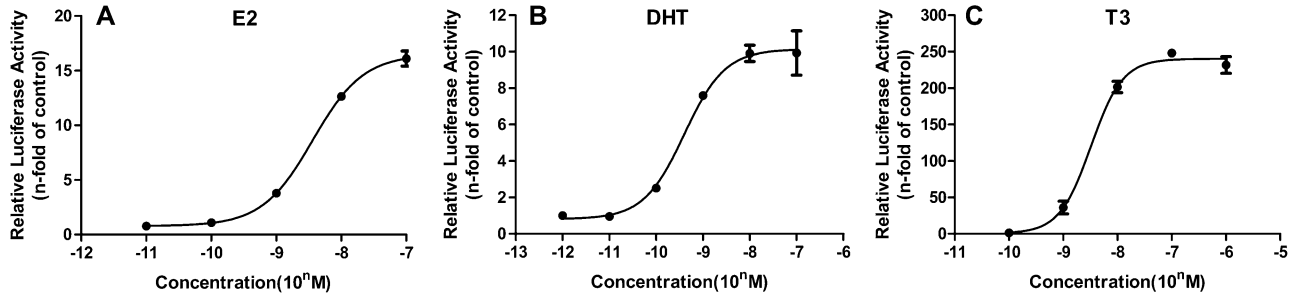

FIG. 2. The estrogenic, androgenic, and TH activities of E2 (A), DHT (B), and T3 (C) in ERα-, AR-, and TRβ-mediated gene assays. (A) CV-1 cells were transfected with pERE-TATA-Luc and rERα/pCI. (B) MDA-kb2 cells were stably transformed with the pMMTV.neo.luc reporter gene plasmid. (C) CV-1 cells were transfected with pUAS-tk-luc and pGal4-L-TR. Cells were treated with increasing concentrations of E2, DHT, and T3 to detect the agonists' activities. Data were presented as mean fold induction compared with vehicle control. Values were mean ± SE of at least three independent experiments.

Renilla luciferase. These indicated that no cytotoxic effects existing at the tested concentrations.

Response of E2 in the ER Assay, DHT in the AR Assay, and T3 in the TR Assay

The estrogenic, androgenic, and TH activities of E2, DHT, and T3 in ERα-, AR-, and TRβ-mediated gene assays are shown in Figure 2. Figure 2A shows the concentration-dependent transactivation of ERα by the natural estrogen E2, which indicated that the assay had appropriate response to E2. The maximal ER activity induced was 16.1-fold of vehicle control at concentration of 10^{-7}M E2. From the concentration-response curve, the EC_{50} value of E2 was 3.73×10^{-9}M.

MDA-kb2 cell line showed appropriate response to DHT that is a known AR agonist. DHT was assayed over concentrations ranging from 10^{-12} to 10^{-7}M. It induced luciferase activity in a concentration-dependent manner (Fig. 2B). For DHT, luciferase activity was significantly increased at 10^{-10}M, and the maximal induction of 9.93-fold of vehicle control was achieved at concentration of 10^{-7}M and remained at the plateau level thereafter. From the concentration-response curve, the EC_{50} of DHT was 3.99×10^{-10}M.

CV-1 cell reporter system showed appropriate response to the natural TR ligand T3. T3-induced luciferase activity in a concentration-dependent manner ranging from 10^{-10} to 10^{-6}M (Fig. 2C). From the concentration-response curve, the EC_{50} of T3 was 2.94×10^{-9}M and the maximal induction of 256.92-fold at concentration of 10^{-7}M and greater as compared with vehicle control.

Estrogenic and Antiestrogenic Activities of Pyrethroids and Their Metabolites

Estrogenic and antiestrogenic activities of tested chemicals are shown in Figure 3. From the concentration-response curve of E2 in the ERα-mediated transactivation assay, we estimated the REC_{20} value of E2 for ERα was 7.12×10^{-10}M. The REC_{20} and RIC_{20} values of each chemical with ERα agonistic and antagonistic effects are shown in Table 2. We found that four of the pyrethroids tested in the ER reporter gene assay had weak estrogenic effects, and these compounds induced estrogenic activity greater than 3.22-fold of the vehicle control (equal to the 20% of the maximum activity of E2) at the concentration of 10^{-5}M (Fig. 3A). Small fold of induction indicated that the tested chemicals had weak estrogenic effect. The relative estrogenic activities of tested chemicals are in following order: cyhalothrin > permethrin > fenvalerate > deltamethrin.

At the same time, six tested chemicals displayed antiestrogenic effects with 1×10^{-9}M E2 in the medium, with a ranking in order of relative decreasing potency of DCCA, cycloprothrin, etofenprox, 3-PBA, cyfluthrin, and permethrin. Among these chemicals, permethrin displayed agonistic effects at concentration from 10^{-8} to 10^{-5}M on the reporter gene expression (Fig. 3A) and also showed weak antagonistic activity at 10^{-6} and 10^{-5}M in the presence of 1×10^{-9}M E2 (Fig. 3B). This indicated that permethrin could act as both an agonist and an antagonist.

Androgenic and Antiandrogenic Activities of Pyrethroids and Their Metabolites

None of tested chemicals had detectable androgenic activity in MDA-kb2 cells in the concentration range from 10^{-9} to 10^{-5}M. The antiandrogenic activity was tested by incubation with 1×10^{-9}M of DHT. Six of the tested pyrethroids and one metabolite suppressed the expression of luciferase. The RIC_{20} value of each chemical with AR antagonistic effect was described in Table 2. Figure 4 illustrated the concentration-response curve of antiandrogenic activities of these chemicals. The relative potencies of their antagonistic activities descended in the following order: deltamethrin > cyfluthrin > fenvalerate > cyhalothrin > cypermethrin > permethrin > 3-PBA.

TR Agonist and Antagonist Activities of Pyrethroids and Their Metabolites

None of tested chemicals showed TR agonistic activity in the TR reporter gene assay. We also tested their inhibitory effect, as showed in Figure 5; all of the tested chemicals except DCCA had antagonist activity. The RIC_{20} value of each

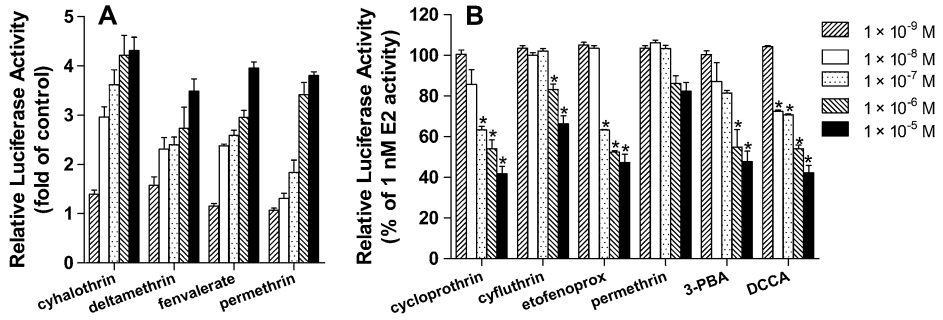

FIG. 3. Estrogenic and antiestrogenic activities of tested pyrethroids and metabolites in the ERα reporter gene assay using CV-1 cells that were transfected with pERE-TATA-Luc and rERα/pCI. (A) Cells were treated with tested compounds in the concentrations from 10^{-8} to 10^{-5}M. Data were presented as *n*-fold induction compared with vehicle control. Values were mean ± SE of three independent experiments. (B) Cells were treated with tested compounds in the concentrations from 10^{-9} to 10^{-5}M with 1×10^{-9}M E2. Values were mean ± SE of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with 1×10^{-9}M E2. *$p < 0.05$, compared with the value of 1×10^{-9}M E2 (100%).

chemical with TR antagonistic effect was described in Table 2. Among these compounds, deltamethrin, etofenprox, permethrin, and tetramethrin exhibited the stronger antagonist activity, with RIC_{20} value less than 1×10^{-8}M.

DISCUSSION

In this study, we characterized the potential ER, AR, and TR activities of nine pyrethroids and two metabolites by using CV-1 cells and MDA-kb2 cell line. The results summarized in Table 2 revealed that these compounds exhibited not only weak estrogenic activities but also antiestrogenic, antiandrogenic, and anti-TH activities via ER, AR, and TR.

In ER-mediated reporter gene assay, cyhalothrin, deltamethrin, fenvalerate, and permethirn could induce minimal luciferase expression. These four pyrethroids had many times lower luciferase induction than E2. Their response trends showed little estrogenic activities, which indicated that these chemicals may become weak estrogen-like substances only at high concentrations. Previous studies found that cyhalothrin showed estrogenic activities in MCF-7 cell proliferation assay (Zhao *et al*., 2008), whereas deltamethrin, fenvalerate, and permethrin exhibited partial agonistic effects in E-screen assay (Chen *et al*., 2002) and pS2 mRNA expression assays (Go *et al*., 1999). Using uterotrophic assay, permethrin could increase relative uterine wet weight (Kim *et al*., 2005). These reports support our current findings that certain pyrethroids were estrogenic. We also found that cycloprothrin, cyfluthrin, etofenprox, permethrin, 3-PBA, and DCCA displayed antiestrogenic effects when tested in competition with 1×10^{-9}M E2. These results were consistent with other reports in which cyfluthrin and 3-PBA

TABLE 2
Agonistic and Antagonistic Activities of Tested Pyrethroid Pesticides and Metabolites on ERα, AR, and TRβ Transcriptional Assays

	ERα		AR	TRβ
Chemicals	REC_{20}[a] (M)	RIC_{20}[b] (M)	RIC_{20}[b] (M)	RIC_{20}[b] (M)
Cycloprothrin	NA	2.30×10^{-8}	NA	5.73×10^{-6}
Cyfluthrin	NA	1.36×10^{-6}	8.84×10^{-8}	5.39×10^{-7}
Cyhalothrin	3.34×10^{-8}	NA	1.18×10^{-6}	3.34×10^{-8}
Cypermethrin	NA	NA	1.64×10^{-6}	8.31×10^{-6}
Deltamethrin	4.14×10^{-6}	NA	1.32×10^{-8}	4.14×10^{-9}
Etofenprox	NA	3.50×10^{-8}	NA	9.85×10^{-9}
Fenvalerate	1.80×10^{-6}	NA	4.48×10^{-7}	2.17×10^{-6}
Permethrin	8.10×10^{-7}	$> 10^{-5}$	1.88×10^{-6}	1.50×10^{-9}
Tetramethrin	NA	NA	NA	2.37×10^{-9}
3-PBA	NA	8.84×10^{-8}	$> 10^{-5}$	4.76×10^{-6}
DCCA	NA	7.23×10^{-9}	NA	NA

Note. NA, not active.

[a]Concentration of the tested chemicals showing 20% of the activity of 1×10^{-7}M E2.

[b]Concentration of the tested chemicals showing a 20% reduction in the activity of 1×10^{-9}M E2 via ERα, 1×10^{-9}M DHT via AR, and 5×10^{-9}M T3 via TRβ respectively.

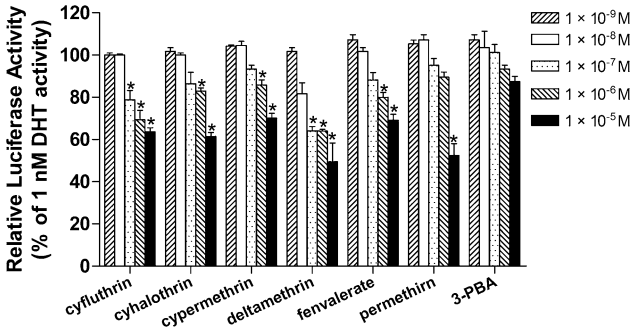

FIG. 4. Antiandrogenic activities of tested chemicals in MDA-kb2 cells that was stably transformed with the pMMTV.neo.luc. Cells were treated with 10^{-9} to 10^{-5}M of tested compounds with of 1×10^{-9}M DHT. Values were mean ± SE of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with 1×10^{-9}M DHT. *$p < 0.05$, compared with the value of 1×10^{-9}M of DHT (100%).

showed antiestrogenic activity in recombinant yeast screen (Tyler *et al*., 2000) and permethrin significantly inhibited the E2-induced MCF-7 BUS cell proliferation (Kim *et al*., 2004).

However, previous reports showed different results in the estrogenic activity of pyrethroids. Kojima *et al*. (2004) found that certain pyrethroids acted as estrogen agonist or antagonist by using Chinese hamster ovary cells. In contrast, none of the tested pyrethroids showed (anti)estrogenic effects on hERα-mediated pathway (Saito *et al*., 2000). Also they (Saito *et al*., 2000) did not find the activity for permethrin that is different from our results. This may be because of the variation in permethrin preparations (Tyler *et al*., 2000). An interlaboratory comparison of receptor gene assays indicated that many factors including the plasmids and cell line must be taken into account (Korner *et al*., 2004). Here we hypothesize that certain pyrethroids may act as weak estrogen agonist and antagonist through ER-mediated pathway in CV-1 cells. The estrogenic effects of some pyrethroids are small comparing to E2, but they still can add to the overall "estrogen load" of the whole body.

The androgens are hormones responsible for male sexual development and maturation, as well as the maintenance of male reproductive function and spermatogenesis. Chemicals possessing antiandrogenic activity can block the natural androgen by binding to AR and thus alter the AR-mediated activation. Using our assay systems, we failed to identify an AR agonist among the tested compounds, but found that six pyrethroids and one metabolite could inhibit the transcriptional activity of DHT. The antiandrogenic activities of deltamethrin and cyfluthrin were more potent than other compounds. Previous studies agreed with our finding: fenvalerate, cypermethrin, permethrin, and their metabolite 3-PBA could act as AR antagonist (Xu *et al*., 2008; Zhang *et al*., 2008). Furthermore, some pyrethroids appeared to competitively inhibit testosterone binding to AR and sex hormone binding globulin (Eil and Nisula, 1990). Significantly reduced plasma testosterone levels were noted in male rats administered deltamethrin for 65 days, which indicated the pyrethroid-mediated endocrine effects (Abd El-Aziz *et al*., 1994). Also, some *in vivo* assays suggested that cypermethrin, deltamethrin, fenvalerate, and permethrin may alter reproductive or endocrine function and adversely affect semen quality in animals (Elbetieha *et al*., 2001; Yousef *et al*., 2003; Zhang *et al*., 2007). In humans, recent studies among Chinese men had suggested a close relationship between pyrethroids metabolite concentrations in urine and reduced sperm concentration (Xia *et al*., 2008). These reports support that some pyrethroids are capable of disrupting male reproductive system, probably because of the interaction with androgen. However, the animal study results (Andrade *et al*., 2002) suggested that deltamethrin had no androgenic effect in Hershberger assay, which is inconsistent with our finding. Hershberger assay may be affected by endocrine mechanisms other than receptor-mediated mechanisms, and the differences in metabolism and pharmacokinetics between *in vitro* and *in vivo* studies could interpret the conflict. Our results focused on the interaction between pyrethroids and hormone receptors and provided insight into the ligand-receptor mechanism directly.

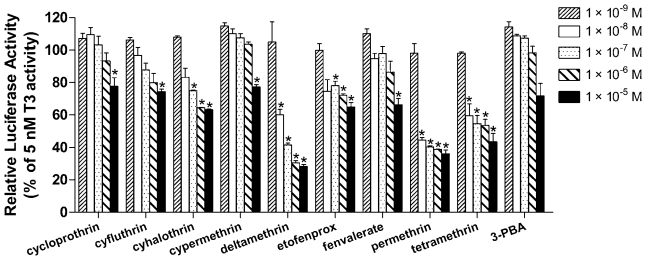

FIG. 5. Anti-TH activities of tested chemicals in TR-mediated reporter gene assay with CV-1 cells transiently transfected with pUAS-tk-luc and pGal4-L-TR. Cells were treated with 10^{-9} to 10^{-5}M of tested compounds with 5×10^{-9}M T3. Values were mean ± SE of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with 5×10^{-9}M of T3. *$p < 0.05$, compared with the value of 5×10^{-9}M T3 (100%).

A few studies on the effects of pyrethroids on TH regulation have been reported. Serum levels of the THs T3 and T4 were significantly decreased in mice administered with fenvalerate (Maiti and Kar, 1998). Research on pyrethroids found that bifenthrin and lambda cyhalothrin could suppress thyroid secretion and inhibit growth rate in young adult rats during 21-day treatment (Akhtar *et al*., 1996). However, these *in vivo* assays investigated the effects of pyrethroids only on thyroid system; their disruptive effects on the binding of THs to their receptors (TR) and subsequent regulatory reaction were rarely studied.

We established a TR-mediated reporter gene assay to screen chemicals that affect the T3-induced transcriptional activation of TR. TR contains a conserved carboxy terminus of LBD that is necessary for transcriptional activation. In order to get vector pGal4-L-TRβ that was used in our TR reporter gene assay, the DNA fragment corresponding to LBD of TRβ1 was fused in frame downstream to the yeast Gal4 DBD. Once activated by

ligand binding, the TR fusion protein activated transcription via a Gal4 response element (UAS) that was engineered upstream of luciferase gene. This assay showed high sensitivity and was used by other researchers before (Moriyama *et al*., 2002, 2007). Some researches (Miyamoto *et al*., 2001; Tagami *et al*., 1997; Tone *et al*., 1994) provided evidences that Gal4-TR possessed similar T3-binding activity as the wild-type receptor and thus could test TR-mediated effects effectively. These results demonstrate that Gal4-TR chimera does not lose its function of binding to ligands and acts as wild-type receptors when binding to a GAL4 DNA-binding site. Using TR reporter gene assay, we found that the tested chemicals except DCCA suppressed the transactivity of reporter gene. However, this assay just focused on the possible effects of pyrethroids on the binding of TH to TR, it did not include the reaction between TR with TH response element and other coregulators. The results we got are part of the disruption effects of pyrethroids on TH. As TH is a critical regulator of vertebrate development, metabolism and homeostasis, the disrupting effects caused by pyrethroids should be of great concern.

Pyrethroids are reported to degrade rapidly in the environment and the body (Miyamoto, 1976). The most common metabolite is 3-PBA, a nonspecific urinary metabolite of many pyrethroids including cyhalothrin, cypermethrin, deltamethrin, fenvalerate, and permethrin. Another common metabolite is DCCA, the metabolite for cyfluthrin, cypermethrin, and permethrin. In the present study, both 3-PBA and DCCA showed antiestrogenic effects with potencies of approximately 100-fold and 1000-fold greater than that of their parent pyrethroids such as cyfluthrin and permethrin, respectively (based on RIC_{20} values). These results suggested that pyrethroid metabolites rather than pyrethroid themselves should attract greater concern regarding their abilities to interact with ER. In AR and TR reporter gene assays, only 3-PBA demonstrated weak antiandrogenic and anti-TH activity, with RIC_{20} exceeding 1×10^{-5} and 4.76×10^{-6}M, respectively. For AR and TR, some metabolites may diminish the hormone-disrupting activity of their parent pyrethroids. In summary, the data presented in our study indicate that some of the major metabolites of pyrethroids may have biologic significance because of their abilities to interact with hormone receptors.

Furthermore, the similarity in chemical structures among pyrethroids may be the primary cause for their hormone receptor activities. The common structure of biphenyl ether moieties play a key role in their properties, and the length and the nature of the substituent groups at the carbon may affect their interaction with hormone receptors. In our study, deltamethrin showed weak estrogenic, antiandrogenic, and anti-TH activities, whereas cypermethrin, which has two chlorine instead of two bromine substituent groups comparing to deltamethrin, displayed antagonistic effects to AR and TR with less potency than deltamethrin. It indicates that brominated pyrethroids have higher potency over chlorinated analogues in the binding affinity to hormone receptors. This was also observed in one other study in which flame retardants tetrabromobisphenol A was a stronger competitor binding to transthyretin than tetrachlorobisphenol A (Meerts *et al*., 2000). What is more, some pyrethroids can produce metabolites such as 3-PBA that possess critical structural characteristic similar to that of E2. These metabolites may antagonize the effect of E2 by blocking its interaction with ER. This may explain its antiestrogenic response of 3-PBA. The relationship between the structure and property of pyrethroids need to be further studied.

Evaluating the classic hormone-mediated activation pathways is critical in understanding biological actions of hormone receptor agonists or antagonists in the environment. The present three *in vitro* reporter gene assays are dependent on ligand-receptor–mediated activation mechanisms. The EC_{50} for E2 in our ER system is 3.73×10^{-9}M that is less sensitive than other systems, such as the T47D-KBluc stable cell line. However, reports from two other laboratories showed that EC_{50} for E2 in ER receptor gene assays were 3×10^{-9} and 9.9×10^{-9}M, respectively, that were close to our finding (Gaido *et al*., 2000; Gould *et al*., 1998). At the same time, the EC_{50} values of 4-alkylphenols and related chemicals measured previously using our ER reporter gene assays were similar to the values gotten in the other laboratories (Gaido *et al*., 2000; Gould *et al*., 1998; Sun *et al*., 2008). It could indicate that our ER reporter gene assay has the potential for screening estrogenic and antiestrogenic activities of chemicals. So our reporter gene assays have high sensitivity, low background noise, and good repeatability and allow reliable evaluation of (anti)hormone activities of chemicals *in vitro*.

In summary, our study confirmed that certain pyrethroids and metabolites are able to have multiple effects on the endocrine system through interfering with ER, AR, and TR. One fact needed to be pointed out is that some pyrethroid products are formulated with synergists, such as piperonyl butoxide and MGK-264, to enhance the pesticide effectiveness. In the future, in *vitro* and *in vivo* studies should consider not only the mechanisms of pyrethroids function alone but also the mixed effects of pesticides and their synergists.

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