Letter

Fipronil, an insecticide, acts as an anti-estrogen via the concomitant down-regulation of ERα and PES1

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ABSTRACT — Endocrine disruptors are ubiquitous in nature. Indeed, some pesticides are not only insect killers, they also function as "endocrine disruptors" in humans and animals; therefore, concern regarding the possible health risks of pesticides for humans is growing. However, few studies have investigated the adverse effects induced by pesticides. One study previously suggested that fipronil reduced the levels of 17β-estradiol (E2), a natural ligand for estrogen receptor α (ESR1, ERα), in female Wistar rat plasma. In the present study, we focused on three relatively novel insecticides: fipronil (a phenyl pyrazole), acetamiprid (a neonicotinoid), and imidacloprid (a neonicotinoid). The effects of these 3 insecticides on the expression of ERα as well as E2/ERα-mediated signaling were examined in an ERα-positive MCF-7 human breast cancer cell line. The results obtained showed that fipronil selectively down-regulated the expression of ERα, and its regulated gene, CDC2, and also that PES1, an upstream signaling molecule for the regulation of ERα, was suppressed by the insecticide. We discussed the potential of fipronil as an anti-estrogen.

Key words: Fipronil, Estrogen receptor α, PES1, Anti-estrogen, MCF-7 cells

INTRODUCTION

Some environmental chemicals exert a number of unwanted effects in humans and animals, including endocrine-disrupting effects (*i.e.*, endocrine disrupters). Chemicals that are capable of functioning as endocrine disruptors are ubiquitous in nature. Previous studies examined the estrogenic and anti-estrogenic effects exerted by environmental chemicals, and revealed that those of the latter, in particular, principally arose from competitive interactions with estrogen receptor α (ESR1, ERα) between 17β-estradiol (E2), a natural ligand, and exogenous chemicals (endocrine disrupters), resulting in the abrogation of E2-mediated physiological actions (Soto *et al*., 1995; Gaido *et al*., 1997; Scippo *et al*., 2004). Since some pesticides are insect killers (selective toxicity) and also function as endocrine disruptors in humans and animals, con-

cern regarding the possible health risks of pesticides for humans is growing. However, few studies have attempted to investigate pesticide-mediated anti-estrogen-like mechanism(s).

A large number of *in vitro* studies have been conducted in order to determine the effects of chemicals on E2-mediated ERα activities using the following methodologies: the E-SCREEN assay (proliferation of ERα-positive MCF-7 cells) and ERα transactivation assay in mammalian/yeast cells (Soto *et al*., 1995; Gaido *et al*., 1997; Scippo *et al*., 2004). Furthermore, the binding capacity of candidates for ERα may be evaluated using a BIAcore surface plasmon resonance biosensor (Rich *et al*., 2002). These lines of study are based on the concept that chemicals evoke their biological activities by interacting with ERα in the presence or absence of E2.

The potential of PES1 (also known as Pescadillo) as a

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molecular target for the effects of environmental chemicals on E2-signaling pathways in MCF-7 cells has recently been suggested (Cheng *et al.*, 2012). In the present study, we focused on three relatively novel insecticides: fipronil (a phenyl pyrazole), acetamiprid (a neonicotinoid), and imidacloprid (a neonicotinoid). Using the MCF-7 human breast cancer cell line as a model that expresses ERα and produces E2 (Miki *et al.*, 2007; Takeda *et al.*, 2009), we investigated the effects of these 3 insecticides on the expression of ERα in addition to E2/ERα-mediated signaling. The results obtained showed that fipronil selectively down-regulated the expression of ERα, and its regulated gene, CDC2, as well as PES1.

MATERIALS AND METHODS

Materials and cell culture

Acetamiprid, fipronil, and imidacloprid were purchased from Sigma-Aldrich (St. Louis, MO, USA). E2 was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade, commercially available, and used without further purification. Δ^9-Tetrahydrocannabinol (Δ^9-THC) was isolated and purified from drug-type cannabis leaves using established methods described elsewhere (Takeda *et al.*, 2008). The purity of Δ^9-THC was found to be at least greater than 98% by gas chromatography (Takeda *et al.*, 2008). Cell culture conditions and methods were performed as described previously (Takeda *et al.*, 2012, 2013; Okazaki *et al.*, 2015). Briefly, the human breast cancer cell lines, MCF-7 and MDA-MB-231 (obtained from the American Type Culture Collection, Rockville, MD, USA), were routinely grown in phenol red-containing minimum essential medium α (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 5% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified incubator, within an atmosphere of 5% CO_2 at 37°C. Twenty-four hours before the chemical treatments, medium was changed to phenol red-free minimum essential medium α (MEMα, Invitrogen) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cultures of approximately subconfluence (close to 60% confluence) in a 100-mm Petri dish were used to seed for the experiments on transfection (dual-luciferase assay) and mRNA expression analyses. All of the insecticides tested were prepared in dimethyl sulfoxide (DMSO, cell culture grade). Control incubations contained the equivalent addition of DMSO.

Transfection and luciferase reporter assay (dual-luciferase assay)

One day prior to transfection, MCF-7 cells (ERα-positive) and MDA-MB-231 cells (ERα-negative) were seeded (5×10^4 cells/well) on 24-well plates containing MEMα. The transfection of each expression plasmid was performed using Lipofectamine® LTX with PLUS™ reagent (Invitrogen) according to the manufacturer's instructions. DNA mixtures containing 300 ng of the (estrogen-responsive element, ERE)$_3$-Luc plasmid were co-transfected with 2 ng of the *Renilla* luciferase reporter plasmid (pRL-CMV) in the plates. In the experiments using MDA-MB-231 cells, an expression plasmid carrying a human ERα cDNA (100 ng) was also transfected. Cells were washed with phosphate-buffered saline 24 hr post-transfection and changed to phenol red-free MEMα supplemented with 5% dextran-coated charcoal-treated serum, followed by the respective chemical treatments being tested. In the experiments using MCF-7 cells, 1 nM E2 was treated 4 hr prior to chemicals treatment. After 24 hr, cell extracts were then prepared using 100 µL of passive lysis buffer (Promega, Madison, WI, USA), and 20 µL was then applied to the firefly luciferase and *Renilla* luciferase assays (Dual-Luciferase Reporter Assay System, Promega) using the GloMax®-Multi Detection System (Promega). The ratio of firefly luciferase activity (expressed from reporter plasmids) to *Renilla* luciferase activity (expressed from pRL-CMV) in each sample served as a measure of normalized luciferase activity.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) analysis

Total RNA was prepared from MCF-7 cells using the RNeasy kit (Qiagen, Inc., Hilden, Germany) and purified by RNeasy/QIAamp columns (Qiagen, Inc.). In the real-time RT-PCR analysis on ERα, CDC2, ERβ, PES1, and β-actin, cDNA was prepared via RT of total RNA using the ReverTra Ace® qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Real-time quantitative RT-PCR assays were performed with FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) and LightCycler® Nano (Roche Diagnostics, Mannheim, Germany). The primers for PCR on human ERα, CDC2, ERβ, PES1, and β-actin were taken from previous studies (Cheng *et al.*, 2012; Takeda *et al.*, 2013). The reaction conditions for all mRNA were 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, at 58°C for 10 sec, and 72°C for 15 sec. ERα, CDC2, ERβ, and PES1 mRNA levels were normalized to the corresponding β-actin mRNA levels.

Data analysis

Differences were considered significant when the *P* value was calculated to be less than 0.05. A data analysis of differences among multiple groups was calculated using Dunnett's test. Calculations were performed using Statview 5.0J software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

We performed studies based on the experimental scheme shown in Fig. 1A (ERα activity and expression analyses). To investigate whether insecticides (acetami-
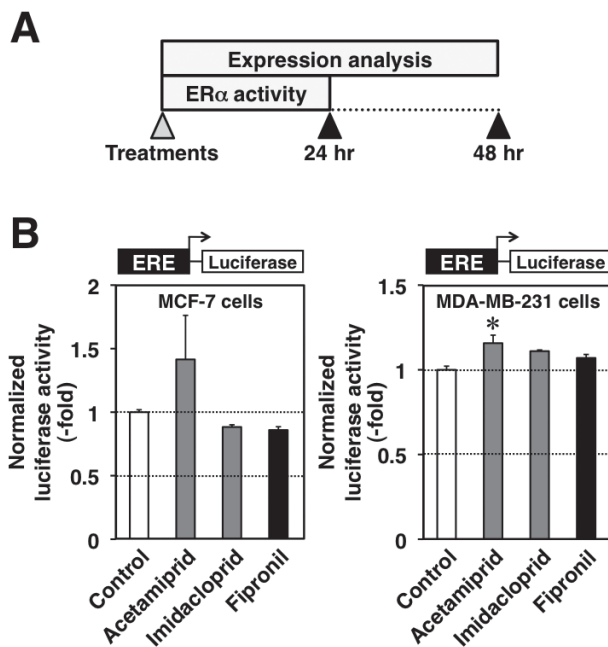

Fig. 1. Experimental scheme (A) and effects of three insecticides (fipronil, acetamiprid, and imidacloprid) on estrogen signaling in ERα-positive MCF-7 cells and ERα-negative MDA-MB-231 cells (B). MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. MDA-MB-231 cells were transiently transfected with an ERE-luciferase reporter plasmid in combination with a human ERα expression plasmid. After transfection, MCF-7 cells and MDA-MB-231 cells were treated with the three insecticides being tested (25 μM) in the presence of E2 or absence of E2, respectively. After 24 hr, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as a fold induction from the vehicle-treated control (indicated as 1), as the mean ± S.E. (*n* = 3). *Significantly different (*P* < 0.05) from the vehicle-treated control.

prid, imidacloprid, and fipronil) behaved as an ERα antagonist and/or an agonist, two experimental systems were employed; ERα-positive MCF-7 cells in the co-presence of E2 (*i.e.*, antagonist) and ERα-negative MDA-MB-231 cells transfected with an expression plasmid containing an ERα cDNA in the absence of E2 (*i.e.*, agonist). As shown in Fig. 1B (left panel), E2-mediated transcriptional activities through ERα in MCF-7 cells were not significantly affected by the three insecticides being tested at 25 μM for 24 hr. In addition, failure of activation was seen in the case with imidacloprid and fipronil in the experiments using MDA-MB-231 cells over-expressing ERα (right panel); however, acetamiprid stimulated the ERα activities (1.16-fold), but it was thought to be a modest extent owing to lack of modulation of CDC2 expression (*See* Fig. 2B). We principally compared the effects of insecticides at 25 μM based on our previous findings to the efficacy of the positive compound, Δ^9-THC (Takeda *et al.*, 2013; Takeda, 2014). As expected, Δ^9-THC inhibited the activity of E2-mediated ERα transactivation in MCF-7 cells; 0.28 ± 0.012 (*n* = 3, *P* < 0.05). We then investigated the effects of the insecticides on ERα and its regulated gene, CDC2 (Lin *et al.*, 2007; Takeda *et al.*, 2013). The concomitant down-regulation of ERα and CDC2 was observed in the fipronil-treated group at 48 hr (Figs. 2A and 2B). It is important to note that although none of the three insecticides negatively modulated ERα activities, the suppression of ERα/CDC2 expression was selectively/significantly greater in the fipronil only group than in the vehicle-treated group. We measured mRNA levels in samples treated with the respective insecticides for 48 hr (*See* Fig. 1A) because the doubling time of MCF-7 cells is approximately 40 hr (Barnes and Sato, 1979; Lu and Serrero, 2001), and CDC2 is a gene that contributes to regulating the cell cycle (Dalton, 1992). The results of experiments using the positive compound Δ^9-THC revealed a good correlation between reductions in ERα transcriptional activities at 24 hr and decreases in CDC2 mRNA levels at 48 hr in MCF-7 cells (Takeda *et al.*, 2013). Accumulating evidence suggests that the second ER, ERβ (the expression of which in MCF-7 cells is maintained at lower levels than that of ERα) functions as a suppressive molecule for the transcriptional activity of ERα via the formation of a less active ERα/ERβ complex than that of ERα/ERα when simultaneously expressed in cells (Cowley *et al.*, 1997; Pettersson *et al.*, 1997). Since the silenced expression of ERβ was stimulated for at least 24 hr by Δ^9-THC (Takeda *et al.*, 2013; Takeda, 2014) (data not shown), the difference observed between Δ^9-THC and fipronil in the modulation of the E2-driven transcriptional activity of ERα may be attributed to the up-regulation
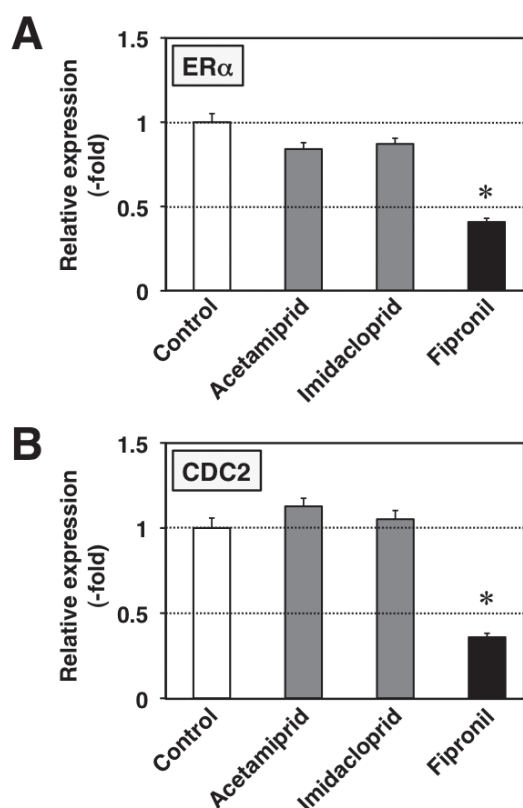
A

B

Fig. 2. Effects of three insecticides (fipronil, acetamiprid, and imidacloprid) on ERα and CDC2 mRNAs in MCF-7 cells. Real-time RT-PCR analyses of (A) ERα and (B) CDC2 in MCF-7 cells 48 hr after the treatment with vehicle or the insecticides (25 μM). Data are expressed as a fold induction from the vehicle-treated control (indicated as 1), as the mean ± S.E. ($n = 3$). *Significantly different ($P < 0.05$) from the vehicle-treated control.

Fig. 3. Effects of three insecticides (fipronil, acetamiprid, and imidacloprid) on PES1 mRNAs in MCF-7 cells. Real-time RT-PCR analyses of PES1 in MCF-7 cells 48 hr after the treatment with vehicle or insecticides (25 μM). Data are expressed as a fold induction from the vehicle-treated control (indicated as 1), as the mean ± S.E. ($n = 3$). *Significantly different ($P < 0.05$) from the vehicle-treated control.

of ERβ.

A recent study reported that PES1, an E2-inducible gene that is strongly expressed in breast cancer, regulates the expression of ERα by modulating mRNA/protein levels (Cheng *et al*., 2012). As is clearly shown in Fig. 3, the expression of PES1 was selectively decreased by fipronil, suggesting that this insecticide targets PES1 in order to down-regulate the expression of ERα in MCF-7 cells. We were unable to provide rational evidence for why only fipronil caused this abnormality; however, a previous study investigated the hydrophobic constant (Log *P*) values of these insecticides, and showed that fipronil had the largest value of 3.75, while those of the other neonicotinoids, acetamiprid and imidacloprid, were 0.80 and 0.57, respectively (Bonmatin *et al*., 2015). Although this may explain the discrepancy described
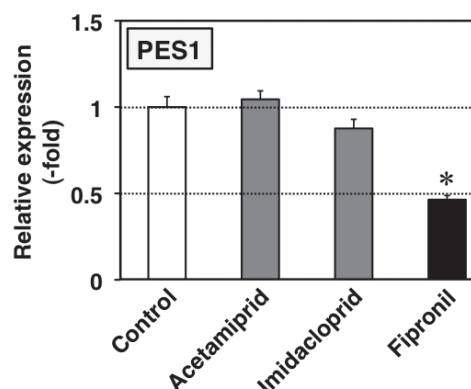
above, at least in part, the Log *P* value of Δ⁹-THC was determined to be 6.97 (Thomas *et al*., 1990).

The results of the present study suggest that fipronil functions as an anti-estrogen in human breast cancer MCF-7 cells by co-down-regulating ERα/PES1, but not (possibly) by targeting ERα as a ligand. Fipronil was previously shown to reduce plasma E2 levels in female Wistar rats (Ohi *et al*., 2004); therefore, the potential of fipronil as an anti-estrogen is strengthened by the results obtained herein.

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