INDUCTION AND INHIBITION OF AROMATASE (CYP19) ACTIVITY BY VARIOUS CLASSES OF PERSISTENT PESTICIDES IN H295R HUMAN ADRENOCORTICAL CARCINOMA CELLS

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Introduction

There is increasing evidence that certain environmental contaminants have the potential to disrupt endocrine processes, which may result in reproductive problems, certain cancers and other toxicities related to (sexual) differentiation, growth and development. Current research has focused mainly on potential interactions with sex hormone receptors, particularly the estrogen receptor. However, other mechanisms of potential interference with endocrine functions exist, including effects on enzymes involved in steroid hormone synthesis and metabolism. A useful bioassay to screen for such interferences is the H295R human adrenocortical carcinoma cell line, which expresses numerous steroidogenic enzymes (1,2). Several classes of (relatively) persistent chlorinated chemicals, namely DDT and several metabolites, and a number of imidazole-type fungicides, suspected or shown to have the capacity to interfere with steroidogenesis (3-5), were screened in the H295R cell line for potential effects on the catalytic activity and mRNA expression of aromatase (CYP19), the key enzyme involved in the biosynthesis of estrogens from androgens.

Materials and Methods

Cell culture conditions. H295R cells from the American Type Culture Collection (ATCC # CRL-2128) were grown under culture conditions published previously (2,6). Cells, in 24-well plates containing 1 ml medium per well, were exposed to the test compounds (0-100 µM) (Riedel deHaen, Germany), administered from 1000-fold stock solutions in 1 µl dimethyl sulfoxide (DMSO). Negative control cells were exposed to DMSO. Positive control cells for induction received 100 µM of 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) dissolved in medium containing 0.1% DMSO. Positive controls for inhibition received various concentration of 4-hydroxyyandrostenedione (4-HA) a potent and selective inhibitor of aromatase. After a 24 h exposure, cells were prepared for RNA analysis, aromatase assay or cytotoxicity assays (MTT reduction and neutral red uptake tests).

RNA isolation and amplification. RNA was isolated using the RNA Insta-Pure System (Eurogentec, Belgium) and stored at -70°C. Reverse-transcriptase polymerase chain reactions (RT-PCRs) were performed using the Access RT-PCR System (Promega, USA). The RNA isolation and RT-PCR amplification conditions were published previously (6). Amplification products were detected using agarose gel electrophoresis and ethidium bromide staining and quantified using a FluorImager (Molecular Dymanics, USA).

Aromatase assay. The catalytic activity of aromatase was determined by the ^3H_2O-release assay (7) with modifications. Cells were exposed to 54 nM 1β-^{3}H-androstenedione (New England Nuclear Research Products, USA) dissolved in serum-free culture medium for 1.5 h. The

specificity of $^{3}H_2O$-release was verified by measuring the aromatization product estrone, using a ^{125}I-labeled double-antibody radioimmunoassay kit (DSL-8700; Diagnostic Systems Inc, USA), and by using 4-HA, an irreversible inhibitor of the catalytic activity of aromatase.

cAMP immunoassay. Intracellular cAMP concentrations were determined using an enzyme-linked immunoassay kit (R&D systems, UK) following the instructions of the supplier. Cells were exposed to the test compounds for 4 h prior to the assay.

Results and Discussion

DDT and the metabolites/analogs o,p-DDT and o,p-DDE decreased aromatase activity in H295R cells, after a 24 h incubation (Fig. 1). However, the concentrations that resulted in significantly decreased aromatase activities were toxic to the cells as indicated by a decreased ability of the cells to reduce MTT or actively take up neutral red (data not shown). Therefore, the effects observed on aromatase activity were considered to be non-specific and likely secondary to decreased cell function.

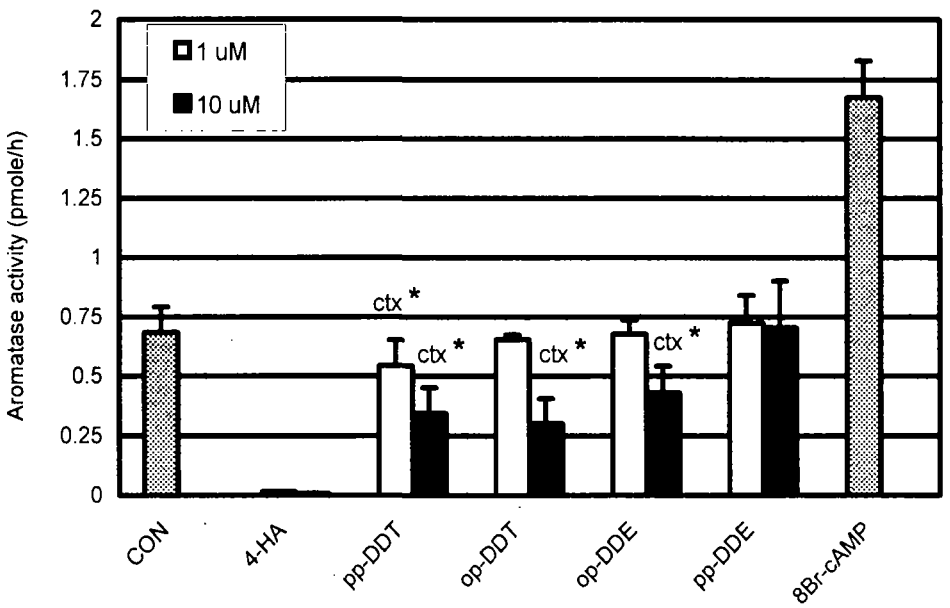

*Fig. 1. Effect on aromatase activity in H295R cells of a 24 h exposure to various DDT metabolites/analogues. CON was DMSO; 1 μM 4-HA and 100 μM 8Br-cAMP was used. Ctx = cytotoxicity; * = significantly lower than control (t-test; $p<0.05$).*

Various imidazole-type fungicides decreased aromatase activity in H295R cells (Fig. 2). In the case of imazalil, prochloraz, difenoconazole, penconazole and propiconazole, aromatase inhibition was considered a specific effect as it occurred at concentrations that had no effect on cell function (determined by MTT reduction and neutral red uptake). Enzyme kinetic (Lineweaver-Burke) analysis indicated that difenoconazole, propiconazole and penconazole were competitive inhibitors of aromatase activity, that imazalil and prochloraz were very potent inhibitors of an uncompetitive or 'mixed' nature, and that fenarimol decreased aromatase activity non-specifically as inhibition occurred only at concentrations that were toxic to the H295R cells (data not shown).

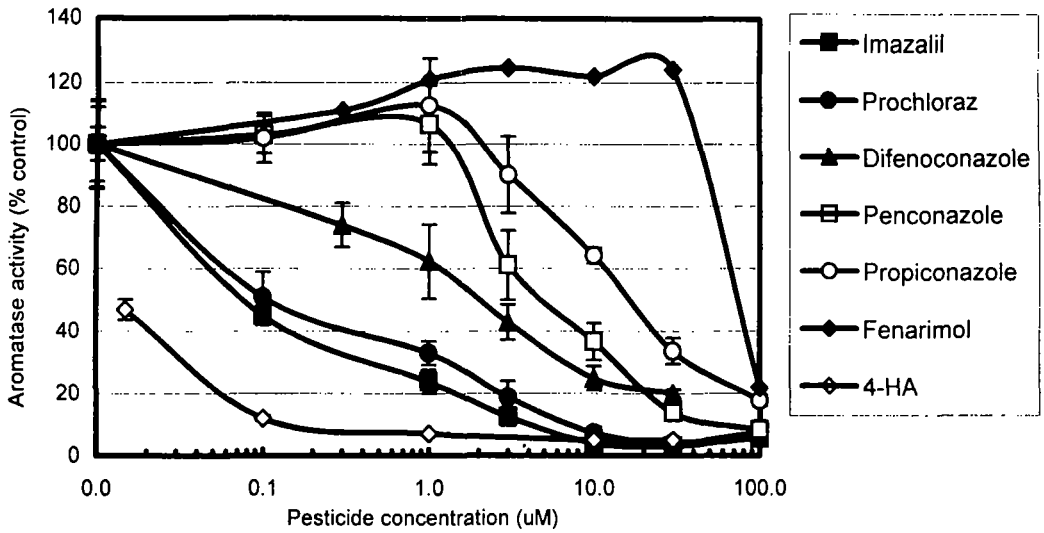

Fig. 2. Inhibition of aromatase activity in H295R cells after a 24 h exposure to various imidazole-type fungicides.

Several imidazole-type fungicides and the structurally related fungicide vinclozolin induced the catalytic activity of aromatase in H295R cells (Fig. 3). Vinclozolin was capable of inducing the activity of aromatase almost 2.5 fold. Diclobutrazole and nuarimol demonstrated a biphasic response with decreased aromatase activities at a concentrations of 100 μM. In the case of diclobutrazole the decrease concurred with a decrease in cell function, but for nuarimol cytotoxicity was not observed. Investigations into the mechanism of induction of aromatase

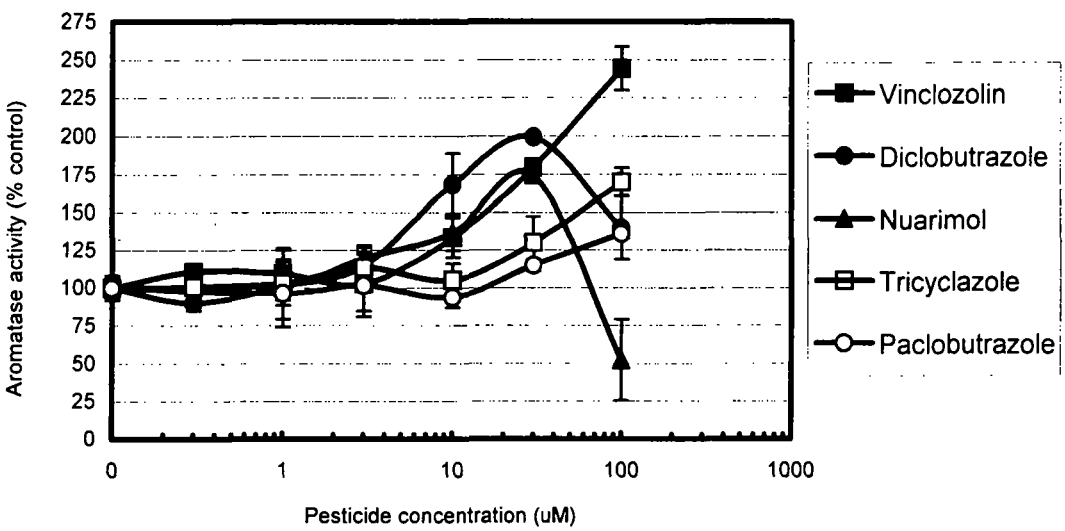

Fig. 3. Induction of aromatase activity in H295R cells after a 24 h exposure to various imidazole-type fungicides.

activity demonstrated that vinclozolin (3, 30, 100 μM) and diclobutrazole (3, 30 μM) were capable of increasing CYP19 mRNA levels concentration-dependently in H295R cells (24 h exposure; data not shown). As the transcription of CYP19 mRNA is regulated by the cAMP-stimulated protein kinase A (PKA) pathway in H295R cells (1), we examined the ability of the inducers vinclozolin, diclobutrazole and an earlier established inducer of aromatase in these cells, atrazine (6), to affect intracellular concentrations of cAMP. Atrazine and vinclozolin (30 μM) increased cAMP levels to about 150% of control, whereas forskolin (a known stimulant of the cAMP synthesizing enzyme adenylate cyclase) increased cAMP levels about 300% (Fig. 4).

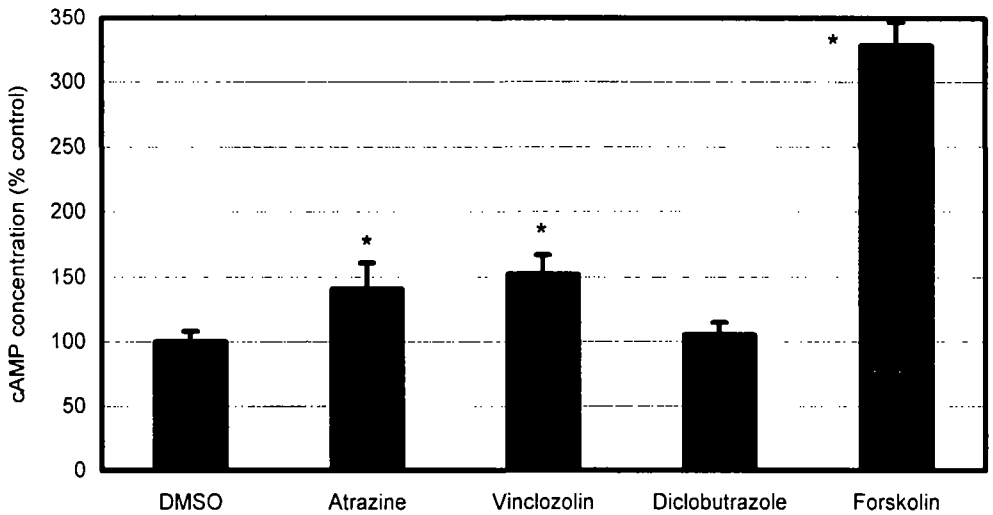

Fig. 4. Effect of a 4 h exposure to atrazine (30 μM), vinclozolin (100 μM), diclobutrazole (30 μM) and forskolin (20 μM) on cAMP levels in H295R cells.

Conclusions

Several classes of (relatively) persistent chlorine-containing (except tricyclazole) pesticides have the capability *in vitro* to interfere with the enzyme aromatase, which is responsible for the conversion of estrogens to androgens in steroidogenic tissues. Using the H295R cell line as a convenient screening tool several effects were observed: 1) specific inhibition of the catalytic activity of aromatase through an interaction with the catalytic or other site on the enzyme, 2) induction of the catalytic activity and mRNA expression of aromatase through a mechanism involving increased transcription via the cAMP-stimulated PKA pathway, and 3) non-specific inhibition of aromatase activity through decreased cell function, secondary to cytotoxic injury.

References

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