TOXICOLOGICAL SCIENCES **53,** 278–288 (2000)

Examination of Selected Food Additives and Organochlorine Food Contaminants for Androgenic Activity *in Vitro*

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Received February 23, 1999; accepted August 30, 1999

In order to produce a reporter gene assay for androgenic chemicals, a constitutive expression vector coding for the human androgen receptor and a reporter construct containing the firefly luciferase coding sequence under transcriptional control of the androgen responsive MMTV promoter were cotransfected into the androgen-insensitive human PC-3 prostate carcinoma cell line and stable transfectants selected. One colony of transfectants, PC-3 LUC^{AR+}, was characterized further. 5α-Dihydrotestosterone (DHT) enhanced luciferase activity in a linear fashion for up to 3 days of culture. The Kd for DHT activation was within the range of 25.0–60.0 pM (r^2 values > 0.95). Flutamide competitively inhibited DHT activation (mean Ki value of 0.89 μM). Progesterone, estradiol, dexamethasone, and hydrocortisone were weak agonists (100-fold less effective than DHT) and diethylstilbestrol was without effect. The effects of organochlorine food contaminants (0, 0.1, 1.0, and 10.0 μM) on luciferase activity in PC-3 LUC^{AR+} cells were determined after exposure to the chemical for 18 h in the presence and absence of DHT (50 pM). 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p′*-DDE) induced luciferase activity in the absence of DHT (100μM *p,p′*-DDE equivalent to 50 pM DHT), but in the presence of DHT (50 pM), *p,p′*-DDE acted antagonistically. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, kepone, butylated hydroxyanisole, and butylated hydroxytoluene all partially inhibited activation by DHT (50 pM) but alone had little or no effect. Toxaphene at 10 μM induced luciferase activity in the absence of DHT but decreased cell viability. α- and δ-Hexachlorocyclohexanes (HCH) at 10 μM antagonized the DHT effect, but β-HCH and γ-HCH mirex, photomirex, oxychlordane, *cis*- and *trans*-nonachlor were without effect. Thus, of the chemicals tested, some interact with the human androgen receptor *in vitro* as agonists, others as antagonists, and some as partial agonists/antagonists.

Key Words: **androgen; receptor; pesticides; food additives; human.**

Male reproductive function requires testicular testosterone for spermatogenesis and 5α-dihydrotestosterone (DHT) for androgen target tissue function (Luke and Coffey, 1994; Sharpe, 1994). In androgen-responsive tissues, testosterone or DHT interact with the androgen receptor and cause the transcription of specific genes (Luke and Coffey, 1994). Exposure to chemicals that interfere with the binding of androgens to the androgen receptor results in abnormalities in sexual development (Husman and McPhaul, 1991; Silversides *et al*., 1995; Spencer *et al*., 1991; van der Schoot, 1992). In recent years, concern has been expressed regarding the possible endocrine disruptive capability of chemicals that persist in nature and accummulate in body tissues. Some chemicals are thought to act as estrogen mimics because they antagonize the effect of estradiol *in vitro* by interacting with the estrogen receptor and because their toxicity *in vivo* resembles the effects of premature estrogen exposure (Colborn *et al*., 1993; Cooper and Kavlok, 1997; Safe, 1995; Turner and Sharpe, 1997). Consequently, the developing male reproductive tract is a target for inappropriate xenoestrogen exposure that could lead to infertility later in adulthood. Furthermore, wildlife studies demonstrating impaired reproductive function in environments where levels of xenoestrogens are high (Colborn *et al*., 1993) have stimulated research into their endocrine effects (Bolger *et al*., 1998; Ramamoorthy *et al*., 1997; Sharpe *et al*., 1995; Shekhar *et al*., 1997; Soto *et al*., 1994; 1995;). While it is clear that *in vitro* and *in vivo* assays of estrogenicity have shown that certain chemicals act as estrogens, the possibility that some chemicals may exert their effects by interfering with the binding of androgens to the androgen receptor has received comparatively little attention. However, the fungicide vinclozolin was reported to mediate its developmental effects as an antiandrogen (Gray *et al*., 1994) and *p,p′*-DDE, which is one of the most abundant organochlorine chemicals in the environment and which does not bind to the estrogen receptor (or at least binds very poorly), has been shown to act as an antiandrogen (Kelce *et al*., 1995; 1997). More recently, other environmental estrogens have been shown to be antiandrogenic *in vitro* (Sohoni and Sumpter, 1998). Studies examining androgenic activity have been hampered by the finding that the only human cell line to be androgen responsive (LNCaP prostate cells) has been found to express a mutated androgen receptor that exhibits altered ligand binding affinity and activation characteristics

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(Kokontis *et al*., 1991; Veldscholte, *et al*., 1992). Therefore, to investigate the intriguing possibility that environmental and food contaminants may be acting as antiandrogens, an androgen-responsive reporter gene assay was developed by cotransfecting human PC-3 androgen-insensitive prostatic carcinoma cells with a human wild-type androgen receptor cDNA expression vector and a MMTV-luciferase reporter construct.

MATERIALS AND METHODS

Chemicals and tissue culture materials. The human PC-3 androgen-insensitive prostate cell line was purchased from the American Type Culture Collection, Rockville, MD. The human androgen receptor expression vector pCMV5-hAR, which contains the full cDNA coding sequence for the human androgen receptor constitutively transcribed through the cytomegalvirus (CMV) immediate-early enhancer/promoter region, was the kind gift of Dr. T.R. Brown, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD. The reporter plasmid pMAMneo-luc, containing the luciferase gene under transcriptional control of the androgen- and glucocorticoid-responsive mouse mammary tumour virus (MMTV) long terminal repeat was purchased from Clontech, Palo Alto, CA. Sterile tissue culture plasticware was purchased from Corning Costar Corporation, Cambridge, MA., and Becton Dickinson, Franklin Lakes, NJ, while glass cloning cylinders were purchased from Bellco Glass Inc., Vineland, NJ. Phenol red-free RPMI medium containing glutamine, trypsin/EDTA, G418, and neutral red were obtained from Gibco, Gaithersburg, MD. Fetal bovine serum was purchased from ICN, Costa Mesa, CA, and charcoal/dextran, tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA), glycerol, Triton X-100, butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA), were purchased from Sigma, St. Louis, MO. Luciferase assay reagent was purchased from Promega, Madison, WI. Technical toxaphene was purchased from Hercules Powder, Wilmington, DE. Chlordane, *cis*- and *trans*-nonachlor and oxychlordane were from Radian International, Austin, TX. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, 99.5% pure by gas chromatography) was a gift from Dr. A. E Pohland, U.S. FDA, Washington, DC. Hexachlorocyclohexanes (α, β, γ, δ), *p,p′* DDE, kepone, and mirex were obtained from the United States Environmental Protection Agency, Research Triangle Park, NC. Photomirex was prepared by Dr. Ih Chu (Health Protection Branch, Health Canada) and was 97% pure. 5α-DHT and estradiol-17β were purchased from Steraloids Inc., Newport, RI and were recrystallized before use. Hydrocortisone and diethylstilbestrol (DES) were from Sigma, St.Louis, MO, dexamethasone was from Aldrich, Milwaukee, WI, and [1,2,4,5,6,7–^{3}H]-5α-DHT (123 Ci/mmol) was from Dupont-NEN, Boston, MA.

Cell culture and transfection. Human PC-3 cells were maintained in 75 cm^2 tissue culture flasks containing 15 ml RPMI medium supplemented with 10% fetal bovine serum stripped of steroids by charcoal/dextran (Borras *et al*., 1994) and gentamycin. Cells were passaged by trypsinization as required.

Cells were transfected using the calcium phosphate:DNA coprecipitation technique of Graham and van der Eb (1973). Briefly, PC-3 cultures were trypsinized, 250,000 PC-3 cells seeded into 60-mm tissue culture dishes, and the cultures allowed to attach overnight. The next day, cultures were cotransfected with pCMV5-hAR (5 μg per plate) and pMAMneo-luc (0.05 μg per plate), the cultures incubated (4 h), and given a 15% glycerol shock. The transfected cells were allowed to recover for 2 days and were then trypsinized and transferred to 100-mm tissue culture dishes. Stable transfectants were selected for G418 (a neomycin analogue) resistance. Resistant colonies were surrounded using cloning cylinders, isolated by trypsinization, and amplified in 24-well tissue culture plates. When confluent, the cells were split into triplicate plates and when again confluent, 1 nM DHT was added to one plate and incubation continued for 24 h. The cells from one of the untreated plates were then lysed in lysis buffer (100 μl, 25 mM Tris-phosphate, pH 7.8, 2mM CDTA, 10% glycerol, 1% Triton-X100) as well as those from the DHT treated plates, and 10-μl aliquots used for luciferase activity. The cells in the remaining plate corresponding to the clone exhibiting a combination of low background fluorescence and high DHT induction ration, designated PC-3 LUC^{AR+}, were chosen for further characterization.

Firefly luciferase assay procedure. PC-3 LUC^{AR+} cells (200,000 in 1 ml medium) were cultured in 24-well plates at 200,000 cells per well for 24 h prior to the addition of DHT (50 pM) and/or chemicals of interest at 0, 0.1, 1.0, or 10.0 μM. Chemicals and steroids were added in 1μl DMSO such that cultures were never exposed to greater than 0.1% DMSO. Eighteen hours later, lysis buffer (100 μl) was added to each well and the plates agitated gently prior to being frozen at –20°C until assay (usually within 48 h). For assay, 10-μl aliquots of cell lysate were transferred to 51 × 12 mm polypropylene luminometer cuvettes (Sarstedt) and light emission measured with a BioOrbit (Turku, Finland) luminometer after injection of 40 μl luciferase assay reagent. Each of the chemicals of interest was examined in at least three separate assays.

The kinetic characteristics of the activation of luciferase activity by DHT in cells were investigated using a range of DHT concentrations (0, 5, 25, 50, 75, 100, 250, and 500 pM). Inhibition of the effect of DHT (0, 5, 25, 50, 75, 100, 250, and 500 pM) was examined using flutamide at 0, 0.25, 1.0, and 10.0 μM. Steroid specificity was assessed by replacing DHT with other steroids (progesterone, estradiol, hydrocortisone, dexamethasone, DES, each at 100, 250, 500, 750, 1000, 2000, and 5000 pM) and comparing the activation with that seen with DHT.

Androgen receptor assays. The number of specific DHT binding sites per PC-3 LUC^{AR+} cell was assessed using modifications of both whole cell (Olea-Serrano *et al.*, 1985) and cytosolic (Green *et al.*, 1986; 1988) assays with nonspecific binding determined by parallel assays using a PC-3 clone in which only the luciferase reporter had been transfected (PC-3 LUC$^{AR–}$). For the whole cell assays, cells were grown in 24-well plates until confluent, including three wells for the determination of cell number. On the day of the experiment, the wells allocated for cell number determination were trypsinized and the cells counted by haemocytometer. For receptor binding measurement in the remaining wells, the medium was replaced and cells incubated in the presence of 0.26–163 nM ^{3}H-DHT for 1 h. After incubation, the medium was removed and the monolayer washed three times with ice-cold PBS. Bound DHT was extracted from the cells by incubation in 250 μl ethanol at room temperature (20 min). Radioactivity in the extracts (200-μl aliquots) was quantified by scintillation counting and specific binding calculated by subtracting the radioactivity per cell associated with PC-3 LUC$^{AR–}$ cells from that found with PC-3 LUC^{AR+} cells. The maximal specific binding per cell was determined by subjecting a plot of the specific radioactivity bound per cell against DHT concentration to one site binding hyperbola nonlinear regression analysis and Scatchard analysis of DHT bound/free against DHT bound (Sigmaplot 4.0, SPSS Inc, 1997, San Rafael, CA) The receptor number was then calculated by taking into account the DHT specific activity and Avogadro's constant.

The second method involved a radioreceptor assay of the cytosol prepared by ultracentrifugation of homogenized PC-3 LUC^{AR+} as well as PC-3 LUC$^{AR–}$ cells. Cells from 10–100 mm dishes were trypsinized, combined, counted, and lysed by 50 strokes in a glass homogenizer on ice in 1 ml of 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 2mM DTT, 50 mM NaCl, 0.3 mM PMSF, 10 mM Na molybdate. After the cytosol was cleared by centrifugation (10,000 × *g*, 15 min, 4°C), glycerol was added to the supernatant to 10% final concentration. Aliquots (50 μl) were taken for hormone binding and incubated with 0–100 nM ^{3}H DHT (0°C, 18 h). Dextran-coated charcoal was added (0.5% in 10 mM Tris-HCl, pH 7.5, 100 μl), the samples incubated on ice (15 min) to remove free steroid, and the samples centrifuged to sediment the charcoal. Bound steroid was measured in 100 μl of supernatant by scintillation counting, specific binding calculated after subtraction of nonspecific binding, and the number of sites per cell determined as for the whole cell assay.

Neutral red assay for toxicity. The effects of the chemicals of interest on cell viability were assessed by staining parallel cultures with neutral red using a modified technique of Borenfreund and Puerner (1985). Briefly, 24 h after

chemical addition, neutral red solution (4 μl) was added per well to a final concentration of approximately 52 μg/ml, and the cultures incubated (1 h). The neutral red- containing medium was removed, the wells washed with PBS, and solubilization buffer added (50% ethanol, 1% acetic acid; 100 μl/well). After mixing on an orbital shaker (15 min), the plates were read on a Cytofluor fluorescence plate reader (Millipore, Beford, MA; 485 nm excitation, 645 nm emission).

Statistical analyses. Kinetic constants for the induction of luciferase expression by DHT and inhibition by flutamide were determined by a nonlinear least-squares method resident in the graphics software (Graph-Pad Prism, version 1.02, 1994, Graphpad Software Incorporated, San Diego, CA). Slope and intercept replots to determine Ki values were obtained from, respectively, Kd/Vmax vs [I] and 1/Vmax vs [I]. Linear regression analyses of the replots were done to determine the Ki values. Significant effects of the chemicals of interest on luciferase activity were determined by one-way ANOVA (Sigmastat 2.0, Jandel Scientific, 1992, 1995, San Rafael, CA) using arcsine transformed proportionalized data. EC_{50} values were determined from the equations for hyperbolic decay generated by Sigmaplot (Sigmaplot 4.0, SPSS Inc., 1997, San Rafael, CA).

RESULTS

Activation of Luciferase Activity by DHT

The activation of luciferase activity in PC-3 LUC^{AR+} cells by DHT (0, 5, 25, 50, 75, 100, 250, 500 pM) was linear with both time and dose for up to at least 18 h in culture (Fig 1A). Linear regression analysis gave r^2 values that were all > 0.92 except for DHT concentrations of 0 pM ($r^2 = 0.01$) and 5 pM ($r^2 = 0.37$). In fact, after 3 days in culture, the activation by DHT was still linear (data not shown). The kinetic constants for DHT activation were determined after a culture period of 18 h in the presence of the above DHT concentrations. A typical example is shown in Fig 1B, in which the Kd for DHT was 50.4 pM. In four similar studies, the Kd for DHT was between 25.0 and 65.0 pM. The involvement of the androgen receptor in the activation by DHT was demonstrated by the inhibitory effect of flutamide (Fig. 2). In four identical studies, flutamide acted as a pure competitive inhibitor of DHT activation with a mean Ki slope of 0.89 μM (Ki intercept values indicated no effect on the Vmax). Furthermore, PC-3 LUC^{AR-}, PC3 cells, which were transfected with the luciferase reporter construct only, and therefore contained no androgen receptor, did not respond to DHT.

Steroid Specificity of Luciferase Activation

The specificity of androgen receptor activation for DHT is shown in Figure 3. Progesterone, estradiol, and hydrocortisone were weak activators, but only at concentrations that were 100- to 1000-fold greater than DHT. The slight activation by progesterone was not affected by inclusion of the antiandrogen flutamide (Fig. 4). Dexamethasone activated the androgen receptor (Fig. 3) but, again, at 100-fold the DHT concentration. Furthermore, flutamide eliminated the activation by dexamethasone (Fig. 4). Diethylstilbestrol did not activate the androgen receptor at any concentration tested (Fig. 3), but in the presence of DHT (50 pM), DES inhibited the DHT-mediated activation (Fig. 5), the EC_{50} for the inhibition by DES being 0.36 μM.

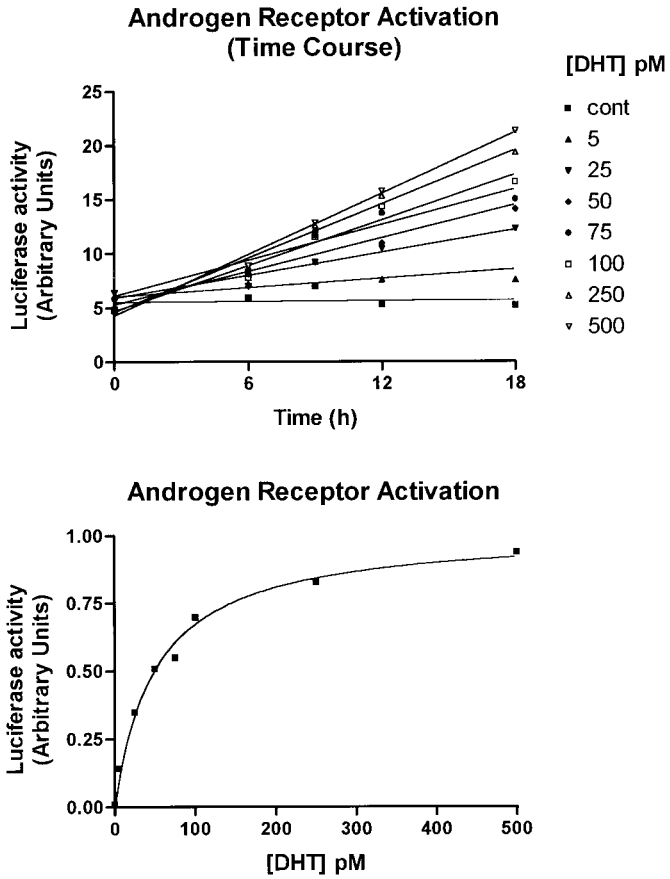

FIG. 1. Time and dose dependence of activation of luciferase activity in PC-3 LUC^{AR+} cells by DHT. PC-3 LUC^{AR+} cells were incubated in the presence of DHT (0, 5, 10, 25, 50, 75, 100, 250, and 500 pM). Incubations were terminated after 0, 6, 12, and 18 h and luciferase activity determined by luminometry described in methods. (A) Linearity with time. (B) Luciferase activity (arbitrary units per hour) vs [DHT]. The Kd for activation in the study shown was 50.4 pM. In similar studies, Kd values were within the range 25–65 pM.

Androgen Receptor Number per Cell

Specific ^{3}H-DHT binding to entire cells revealed that the number of DHT binding sites per cell was 46,500. Control cells into which no androgen receptor had been transfected revealed no specific binding of ^{3}H-DHT (Scatchard analysis revealed a positive slope). In an alternative assessment, when binding of ^{3}H-DHT to cytosolic extracts from a known number of PC-3 LUC^{AR+} cells was determined, the number of specific binding sites was found to be a comparable 30,500 sites per cell.

The Effect of p,p′-DDE on Androgen Receptor Activation

When PC-3 LUC^{AR+} cells were incubated with *p,p′*-DDE (50 and 100 μM), luciferase activity was induced, although not

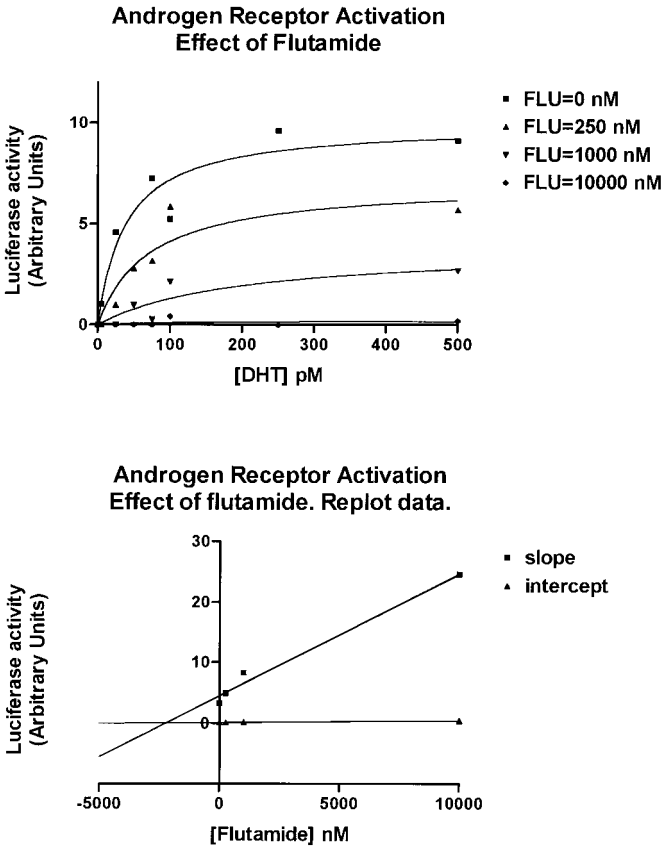

FIG. 2. Effect of the antiandrogen flutamide on the activation of luciferase activity in PC-3 LUC^{AR+} cells by DHT. PC-3 LUC^{AR+} cells were incubated for 18 h in the presence of DHT (0, 5, 10, 25, 50, 75, 100, 250, and 500 pM) and flutamide (0, 250, 1000, and 10,000 nM). (A) Activity vs [DHT] in the presence of flutamide. (B) Slope and intercept replots of the data presented in (A). The Ki from the slope replot was 2.3 μM. The intercept replot was parallel to the x-axis.

as effectively as observed with 50 pM DHT (Fig. 6). However, *p,p′*-DDE concentrations of 1.0 and 5.0 μM partially antagonized (58%) the DHT- mediated activation of the androgen receptor, and higher concentrations completely antagonized the DHT activation (Fig. 6 and Table 1); the luciferase activity measured was the same as in the nonstimulated cells ($p > 0.05$). Cell viability was reduced by *p,p′*-DDE concentrations of 50 and 100 μM (36% and 24%, respectively, compared with control) but in the presence of 1.0, 5.0, and 10.0 μM *p,p′*-DDE, the viability was 100, 89, and 76% compared with control.

The Effect of Toxaphene on Androgen Receptor Activation

Toxaphene at 10 μM activated the androgen receptor (Fig. 7) and this activation could be inhibited by flutamide (10 μM), but this concentration of toxaphene reduced cell viability to 64% of control. Toxaphene had no effect on the DHT-mediated activation (50 pM; Fig. 7 and Table 1).

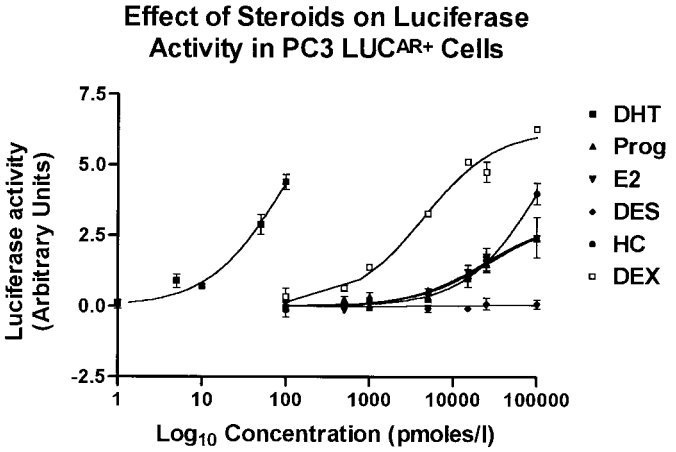

FIG. 3. Steroid specificity of activation of luciferase activity in PC-3 LUC^{AR+} cells. PC-3 LUC^{AR+} cells were incubated for 18 h in the presence of DHT (0, 25, 50, 75, and 100 pM), progesterone, estradiol-17β, diethylstilbestrol, hydrocortisone, and dexamethasone (100, 500, 1000, 5000, 15,000 25,000, 100,000 pM).

The Effect of TCDD, Kepone, BHT, BHA, and Hexachlorocyclohexanes on Androgen Receptor Activation

TCDD, kepone, BHT, and BHA were inactive in the absence of DHT, but all antagonized the DHT activation (50 pM) of the androgen receptor such that at 10 μM the DHT effect was inhibited by at least 50% (Fig. 8 and Table 1). Furthermore, cell viability was not affected by these chemicals except for kepone at 10 μM, which reduced viability to 35% compared with control cells. α-HCH in the absence of DHT had no effect on androgen receptor activation, but at 10 μM antagonized the DHT-mediated activation of the androgen receptor with no effect on cell viability (94% compared with control). However,

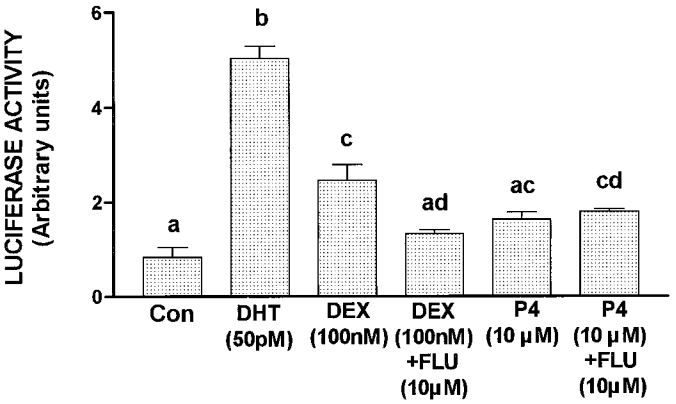

FIG. 4. The effect of flutamide on the activation of luciferase activity in PC-3 LUC^{AR+} cells by progesterone and dexamethasone. PC-3 LUC^{AR+} cells were incubated for 18 h in the presence of DHT (50 pM), progesterone (10 μM), or dexamethasone (100 nM). Flutamide (10 μM), inhibited the activation by dexamethasone but had no effect on the slight activation seen with progesterone. (Bars with differing superscripts are significantly different by ANOVA, $p < 0.05$).

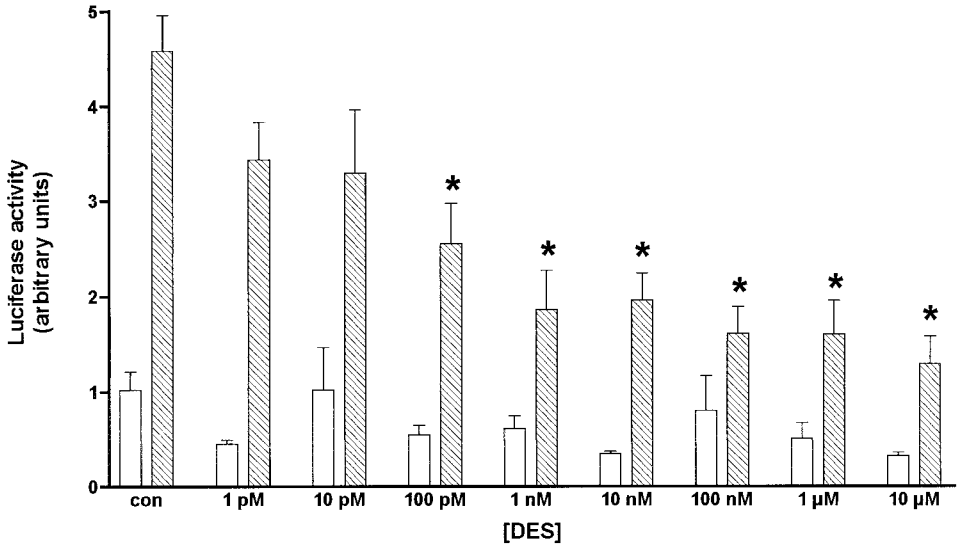

FIG. 5. The effect of DES on DHT-mediated activation of luciferase in PC-3 LUC^{AR+} cells. PC-3 LUC^{AR+} cells were incubated for 18 h in the presence (hatched bars) or absence (open bars) of DHT (50 pM) and DES (1, 10, 100 pM, 1, 10, 100 nM, 1, 10 μM). The EC_{50} for the inhibition by DES was determined to be 0.36 μM (Sigmaplot, hyperbolic decay). * Significantly different compared with DHT (50pM) (ANOVA, $P < 0.05$).

β-HCH, γ-HCH, and δ-HCH were ineffective both in the presence and absence of DHT (50 pM; Fig. 9 and Table 1). Similarly, mirex, photomirex, oxychlordane, *cis*- and *trans*-nonachlor had no effect on androgen receptor activation either in the presence or absence of DHT (50 pM) (data not shown).

DISCUSSION

The characteristics of the transfected PC-3 LUC^{AR+} cells described in these studies are similar to those reported by others (Dai *et al*., 1996; Lustig *et al*., 1994; Sohoni and Sumpter, 1998; Yuan *et al*., 1993) with the exception that the Kd for DHT is somewhat lower (50 pM as opposed to 100 pM). In common with other systems that have been developed, DHT is at least 100-fold more active than nonandrogenic steroids in androgen receptor activation and this effect of DHT can be blocked by the antiandrogen flutamide. PC-3LUC^{AR-} cells that were transfected with the luciferase reporter construct alone did not respond to DHT. When nonandrogenic steroids were assessed for interaction with the androgen receptor, high concentrations of dexamethasone (100-fold greater than DHT)

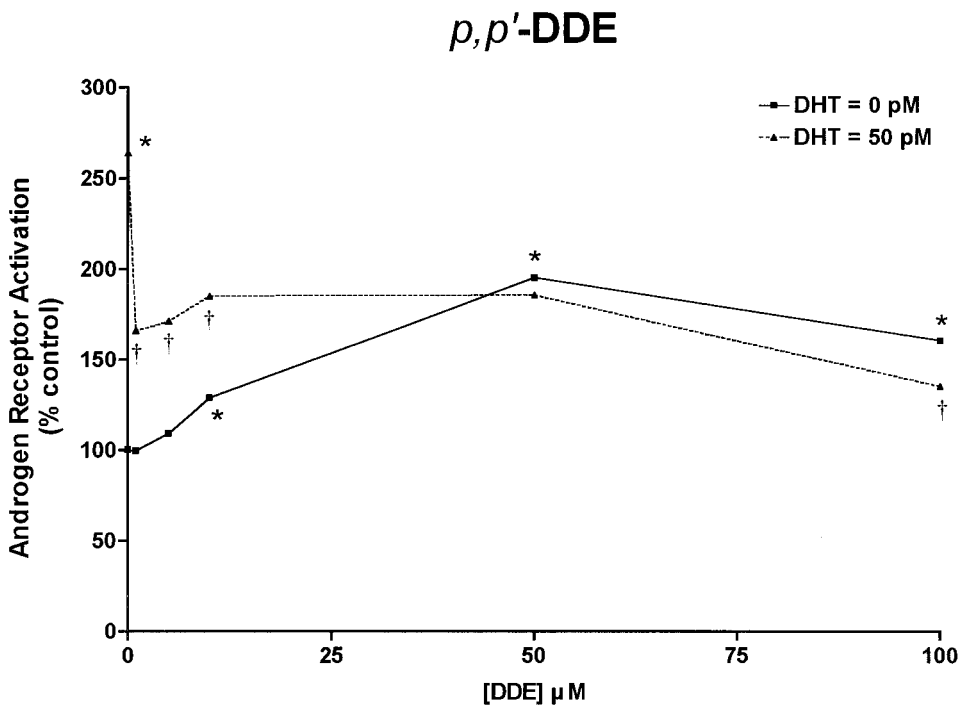

FIG. 6. The effect of *p,p′*-DDE on luciferase activity in PC-3 LUC^{AR+} cells in the presence and absence of DHT. PC-3 LUC^{AR+} cells were incubated for 18 h with *p,p′*-DDE (0, 1, 5, 10, 50, and 100 μM) in the presence or absence of DHT (50 pM). In the absence of DHT, *p,p′*-DDE (10, 50, and 100 μM) significantly activated luciferase activity compared with control (no DHT, no *p,p′*-DDE) ($p < 0.05$). In the presence of DHT, inhibition of the activation by DHT was observed at all *p,p′*-DDE concentrations ($p < 0.05$). * Significantly different compared with control (100%, no DHT, no DDE), † significantly different compared with DHT (50 pM, no DDE) using arcsine transformed proportionalized data from four separate studies.

TABLE 1
EC_{50} Values for the Inhibition of DHT-Mediated Activation of Luciferase Activity

Chemical	EC_{50} (μM)[a]
p,p′-DDE	15.2
TCDD	6.5
BHA	7.6
BHT	5.7
Kepone	6.9
α-HCH	8.2
β-HCH	∞
γ-HCH	∞
δ-HCH	17.9
Toxaphene	1935.0

Note. The data shown in Figures. 6–9 were analyzed using Sigmaplot software to determine the equation for hyperbolic decay; the EC_{50} values were then obtained by substitution.

[a] ∞ indicates no determinable value.

caused increased luciferase activity and this effect was inhibited by flutamide, indicating that the activation was through the androgen receptor. DES alone did not cause luciferase activation but inhibited the response to DHT with an estimated EC_{50} of 0.36 μM. This is in agreement with similar data obtained from competitive binding assays where a Ki of 4.6 μM for DES was determined for the displacement of R1881 from a cytosolic androgen receptor (Kelce *et al*., 1995).

We have used PC-3 LUC^{AR+} cells to investigate the possibility that some organochlorine food contaminants and food additives may interact with the androgen receptor. This was first reported for vinclozolin (Gray *et al*., 1994) and *p,p′*-DDE (Kelce *et al*., 1995) and more recently for DDT and butyl benzyl phthalate (Sohoni and Sumpter, 1998). While *p,p′*-DDE has been found to have poor estrogenic activity (Kelce *et al*., 1995) and vinclozolin appeared to act via the androgen receptor (Wong *et al*., 1995), the other two chemicals have been shown to have estrogenic activity (Soto *et al*., 1995) in addition to androgenic activity. In the present studies, we have confirmed that *p,p′*-DDE interacts with the androgen receptor both as an agonist in the absence of DHT and as an antagonist in the presence of DHT. The agonistic action was observed at high concentrations and was accompanied by poor cell viability. The antagonistic action of *p,p′*-DDE was observed at concentrations as low as 1.0 μM and complete antagonism of the DHT effect was seen at 10 μM. In a radioreceptor assay of the rat prostate androgen receptor, *p,p′*-DDE at 100 μM completely inhibited the binding of DHT but was only partially active in preventing the binding of DHT to epididymal androgen binding protein (Danzo, 1997). Thus, the action of *p,p′*-DDE as an endocrine disrupter would appear to be predominantly at the level of the androgen receptor.

Toxaphene is a persistent environmental contaminant that has estrogenic activity. At 10 μM, toxaphene promotes the proliferation of MCF7 cells and increases the progesterone receptor levels in these cells, but at 1.0 μM toxaphene has no effect on these end points. However, at both 1.0 and 10 μM, toxaphene appeared to inhibit the processing of estrogen receptors (Soto *et al*., 1995). To our knowledge, prior to the present studies, toxaphene has not been examined for interaction with the androgen receptor. We observed that while toxaphene was a poor antagonist, it activated the androgen receptor at 10 μM in the absence of DHT, and although this activation was blocked by flutamide, it was observed when cell viability was reduced. This paradox confounds interpretation, but the present evidence suggests that toxaphene interacts with estrogen receptors and extremely poorly with androgen receptors. As both androgen and estrogen receptors are essential for normal male reproductive development (George and Wilson, 1994; Layman, 1995; Luke and Coffey, 1994; Sharpe, 1995),

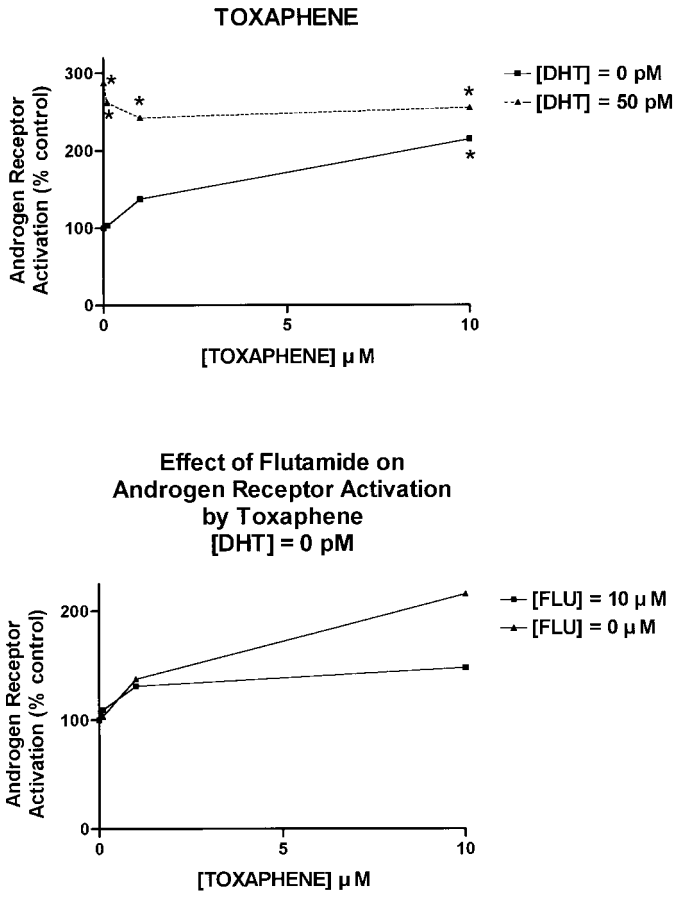

FIG. 7. The effect of toxaphene on luciferase activity in PC-3 LUC^{AR+} cells in the presence and absence of DHT. PC-3 LUC^{AR+} cells were incubated for 18 h with toxaphene (0, 0.1, 1.0, and 10 μM) in the presence or absence of DHT (50 pM). (A) In the absence of DHT, toxaphene (10 μM) significantly activated luciferase activity compared with control (no DHT, no toxaphene) ($p < 0.05$). When DHT was present, toxaphene was without effect. * Significantly different compared with control (100%) using arcsine transformed proportionalized data from four separate studies. (B) Flutamide (10 μM) inhibited the activation of luciferase activity toxaphene.

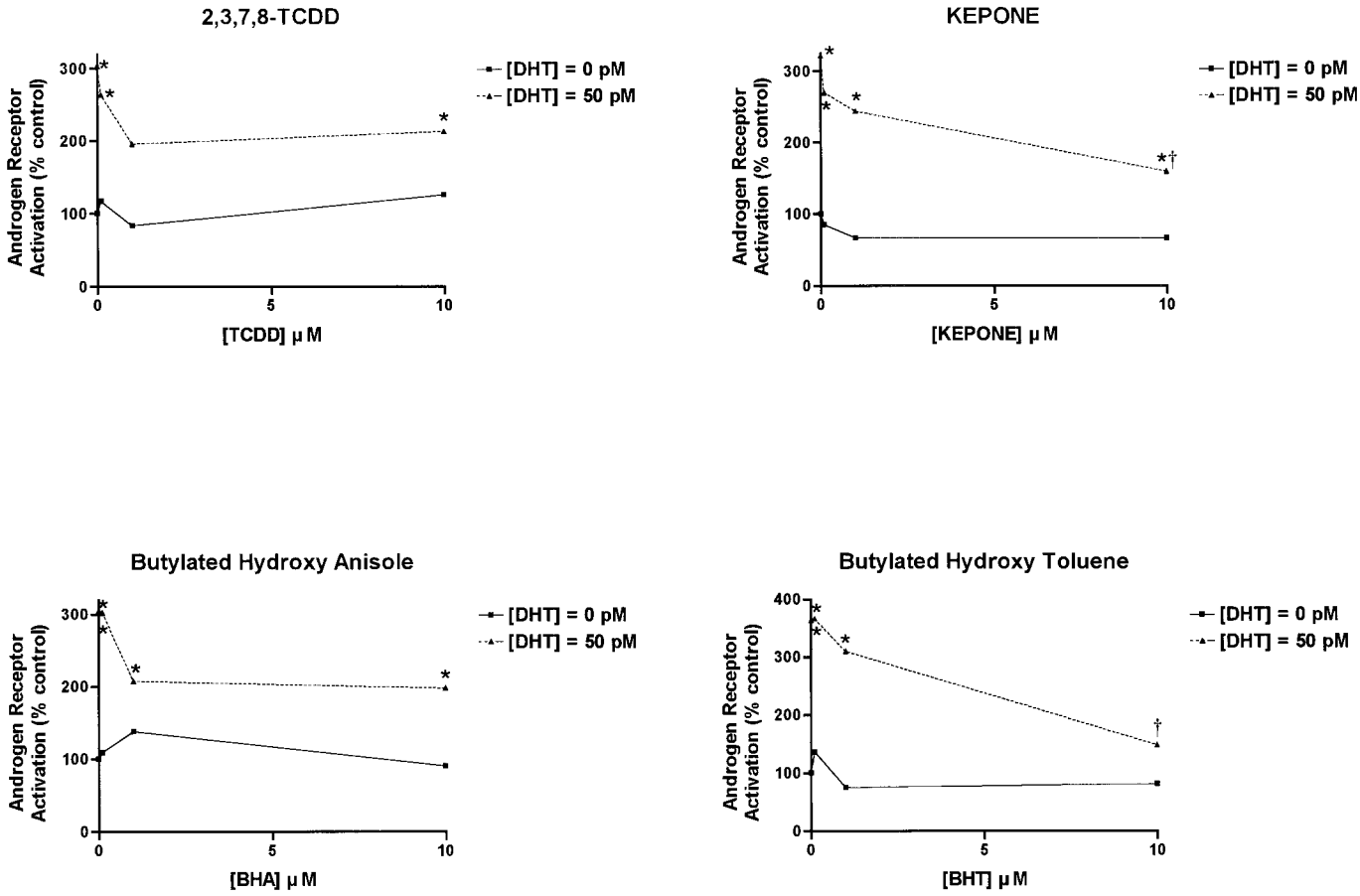

FIG. 8. The effects of (A) TCDD, (A) kepone, (C) BHA and (D) BHT on luciferase activity in PC-3 LUC^{AR+} cells in the presence and absence of DHT. PC-3 LUC^{AR+} cells were incubated for 18 h with the chemical (0, 0.1, 1.0, and 10 μM) in the presence or absence of DHT (50 pM). None of these chemicals activated luciferase activity. Only BHT at 10 μM completely antagonized the activation by DHT. TCDD, kepone, and BHA were partially antagonistic. * Significantly different compared with control (100%, no DHT, no chemical). † Significantly different compared with DHT (50 pM, no chemical) using arcsine transformed proportionalized data from four separate studies.

toxaphene may be able to interfere with estrogen-dependent processes but would be less likely to interfere with androgen-dependent processes.

In contrast to the androgenic effect of toxaphene, TCDD, kepone, BHA, and BHT acted only as partial androgen antagonists in these studies. TCDD has been shown to have endocrine-disruptive effects on the male reproductive system that were considered to be due to antiestrogenic activity or to other mechanisms not related to steroid receptors, such as steroid biosynthesis and gonadotropin responsiveness (Bjerke and Peterson; 1994; Bjerke *et al*., 1994a,b; Gray *et al.*, 1995, 1997; Mably *et al.*, 1992a,b,c; Moore and Peterson, 1988; Peterson *et al*., 1993). Kepone is considered to have estrogenic activity and is able displace estradiol from the estrogen receptor (Bolger *et al*., 1998; Soto *et al.*, 1995). However, an interaction with the androgen receptor appears to be less important, as in the present study the antagonistic effect of kepone was accompanied by a significant reduction in cell viability and in other studies, the ability of kepone to displace androgen from the androgen receptor was only seen at concentrations higher than 50 μM (Kelce *et al*., 1995).

BHA and BHT are used as antioxidants in foods. BHA is considered to be estrogenic due to its promotion of MCF7 cell proliferation (Soto *et al*., 1995) and its ability to stimulate a response in MCF7 cells transfected with an estrogen-regulated luciferase construct (Jobling *et al*., 1995). BHT, on the other hand, had no effect on either of these end points and is not considered estrogenic. It is of interest, therefore, that both BHA and BHT were androgen antagonists in the present studies and that BHT completely inhibited the activation by DHT without having deleterious effects on cell viability. Consequently, BHT could act as an androgen antagonist, but BHA could act as an estrogen and also as an androgen antagonist.

In MCF7 cell proliferation assays, lindane (γ-HCH) was considered to be nonestrogenic (Soto *et al*., 1995). Lindane has the potential to be toxic to the male reproductive system (Dalsenter *et al*., 1996; Prasad *et al*., 1995; Silvestroni *et al*., 1997). Toxicity does not appear to be due to any interaction

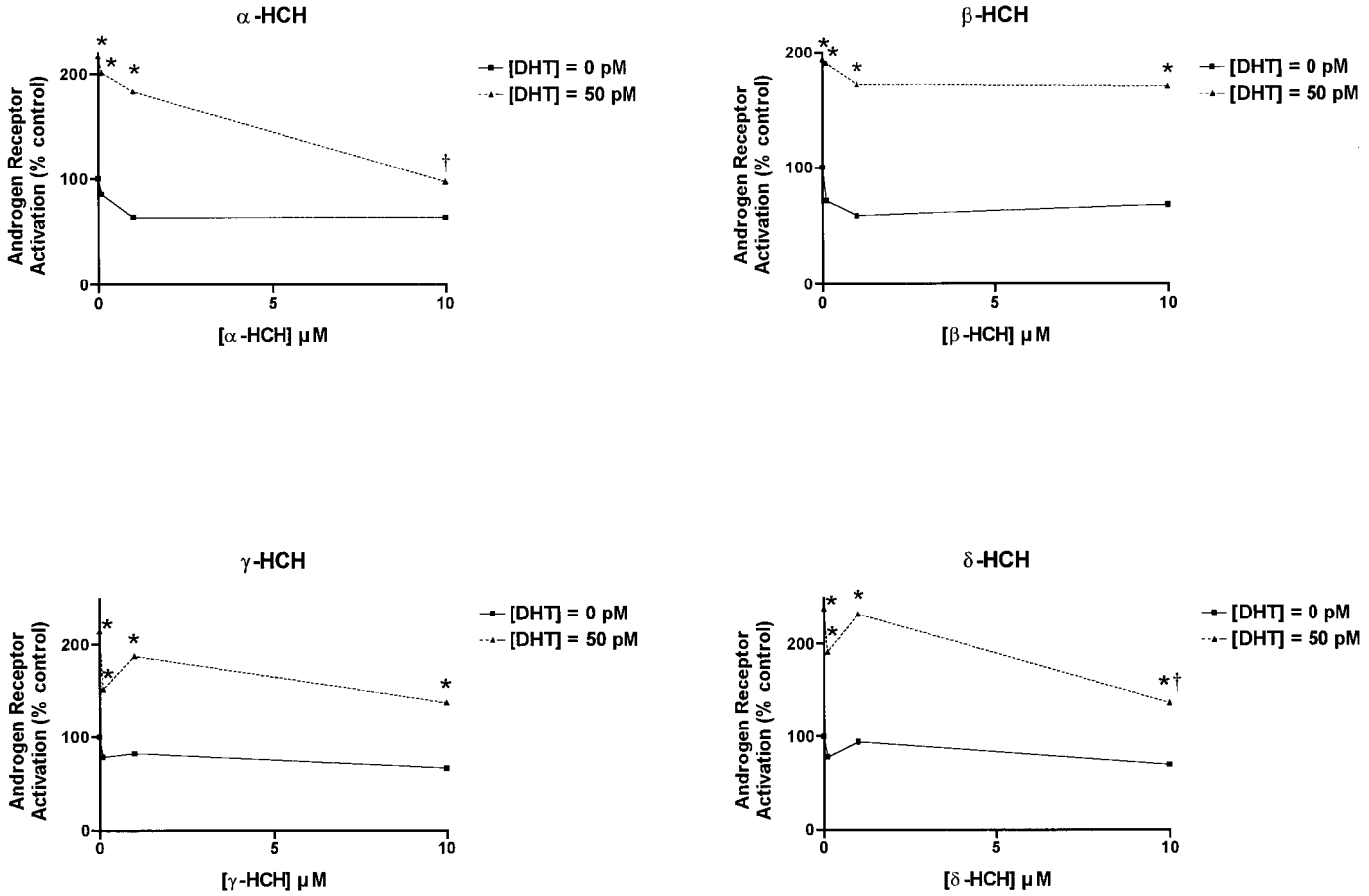

FIG. 9. The effects of hexachlorocyclohexanes on luciferase activity in PC-3 LUC^{AR+} cells in the presence and absence of DHT. PC-3 LUC^{AR+} were incubated for 18 h with the chemical (0, 0.1, 1.0, and 10 μM) in the presence or absence of DHT (50 pM). None of these chemicals activated luciferase activity. Only α-HCH at 10 μM completely antagonized the activation by DHT. * Significantly different compared with control (100%, no DHT, no chemical), † Significantly different compared with DHT (50 pM, no chemical) using arcsine transformed proportionalized data from four separate studies.

with either the androgen receptor (these studies) or with androgen- binding protein, but could possibly arise through its weak affinity for the human SHBG (Danzo, 1997). On the other hand, δ-HCH, which was effective in displacing androgen from androgen- binding protein, had little affinity for the androgen receptor (Danzo, 1997). Of the HCHs, only α-HCH appeared to have any effect on the androgen receptor, and only at a concentration of 10 μM.

Mirex, photomirex, oxychlordane, *cis*- and *trans*-nonachlor exhibited no interaction with the androgen receptor in our system. Mirex is not considered to be estrogenic (Soto *et al*., 1995), nor is technical chlordane, the chlordane constituents *cis*- and *trans*-nonachlor, or the metabolite oxychlordane (Soto *et al*., 1995). To our knowledge, the possibility that these compounds might interact with the androgen receptor has not been investigated previously.

The mechanism of androgen receptor-mediated responses has been the subject of several recent studies. The androgen receptor binds to DNA as a dimer (Langley *et al.*, 1995; Zhou *et al.*, 1995). The possibility that mixed-ligand dimers (e.g., a DHT-AR dimerized with progesterone-AR complex) could lead to blocked gene expression has been proposed to explain mixed agonist/antagonist activity (Maness *et al.*, 1998). In other studies, the type of conformational change in the androgen receptor that occurs on binding ligand has been related to the stability of the complex, which in turn dictates the extent of androgen receptor-dependent gene expression (Doesburg *et al*., 1997; Kemppainen and Wilson, 1996; Kuil and Mulder, 1994, 1995; Zhou *et al*., 1995). However, other possibilities exist for ligands to affect androgen receptor-mediated function that may not be directly related to androgen-receptor binding. Chemicals that interfere with androgen receptor coactivators such as ARA_{55} and ARA_{70} (Fujimoto *et al.*, 1999; Yeh and Chang, 1996) or with the vitamin D or retinoic acid receptors (Zhao *et al.*, 1999) could also result in altered androgen responses. In addition, activation of the protein kinase A pathway can lead to the expression of androgen receptor-dependent genes in the absence of androgen (Sadar, 1999), indicating that interference with the cross-talk between the PKA signal transduction pathway and the androgen receptor could lead to altered gene

expression. Consequently, there are several possibilities for xenobiotics to affect the expression of androgen-dependent genes. Some would be related to the nature of their binding to the androgen receptor, but other mechanisms are possible that would not require direct interaction with the ligand-binding domain of the androgen receptor.

When considering the physiologic consequences of exposure to xenobiotics, it is important to relate the findings of research activities to the levels of human exposure and recommended daily intake levels. In terms of androgen-receptor activation, these studies have shown that *p,p′*-DDE, BHT, kepone, and α- and δ-HCHs exhibited androgen antagonistic actions. For *p,p′*-DDE, estimates of greater than 1 μg/g breast milk fat have been determined in certain indigenous populations (Dewailly *et al*., 1993; Mes, 1993; Newsome *et al*., 1995) and levels greater than 1 μg/l in the cord blood of Inuit newborns have been detected (Canadian Arctic Contaminants Report, 1997). An acceptable daily intake of 0.5 mg/kg/day has been recommended for BHT and no reproductive impairment has been observed in animal studies with doses equivalent or greater than the TDI (Smith, 1984). The chemical structures of mirex, photomirex, and kepone are closely related; whereas mirex and photomirex had no effect on androgen receptor activation, kepone (10 μM) was antagonistic. Environmental levels of kepone have been diminishing since cessation of usage in the late 1970s. Seafoods containing less than 400 ng/g are considered unlikely to cause harm (Faroon and Kueberuwa, 1995). Tolerable daily intake levels of 300 ng/kg/day for α- and δ-HCHs have been recommended in the United States and Canada (Choudhary *et al*., 1994). An estimated intake of 5 ng/kg/day for α-HCH was obtained and human breast milk contains 10–40 ng/g lipid (Canadian Arctic Contaminants Report, 1997). Data for δ-HCH are sparse, but in one study the levels in human breast milk were undetectable (Keewatin Environmental Health Project, 1998). It would appear that, with the possible exception of *p,p′*-DDE, normal human exposure to the chemicals investigated in these studies would be unlikely to cause reproductive dysfunction through antagonism of androgen receptor-mediated events, although other mechanisms could be possible targets for these chemicals.

In conclusion, we report here the development and use of a system to evaluate the interaction between environmental food contaminants and the human androgen receptor in a human prostate cancer cell line. This assay can be used to determine whether a chemical can activate the receptor or antagonize the activation of the receptor by DHT. It is anticipated that this assay will prove useful in the screening of chemicals that are possible endocrine disrupters.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Mme. Carole Whalen and Mr. Georges Angers.

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