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Disruption of Androgen Regulation in the Prostate by the Environmental Contaminant Hexachlorobenzene

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Hexachlorobenzene (HCB) is a persistent environmental contaminant that has the potential to interfere with steroid hormone regulation. The prostate requires precise control by androgens to regulate its growth and function. To determine if HCB impacts androgen action in the prostate, we used a number of methods. Our *in vitro* cell-culture–based assay used a firefly luciferase reporter gene driven by an androgen-responsive promoter. In the presence of dihydrotestosterone, low concentrations (0.5–5 nM) of HCB increased the androgen-responsive production of firefly luciferase and high concentrations of HCB (> 10 μM) suppressed this transcriptional activity. Results from a binding assay showed no evidence of affinity between HCB and the androgen receptor. We also tested HCB for *in vivo* effects using transgenic mice in which the transgene was a prostate-specific, androgen-responsive promoter upstream of a chloramphenicol acetyl transferase (CAT) reporter gene. In 4-week-old mice, the proportion of dilated prostate acini, a marker of sexual maturity, increased in the low HCB dose group and decreased in the high HCB dose mice. In the 8-week-old mice, there was a significant decrease in both CAT activity and prostate weight upon exposure to 20 mg/kg/day HCB. Therefore, *in vitro* and *in vivo* data suggest that HCB weakly agonizes androgen action, and consequently, low levels of HCB enhanced androgen action but high levels of HCB interfered. Environmental contaminants have been implicated in the rising incidence of prostate cancer, and insight into the mechanisms of endocrine disruption will help to clarify their role. *Key words:* androgen receptor, hexachlorobenzene, mice, prostate, steroid hormone. *Environ Health Perspect* 111:461–466 (2003). doi:10.1289/ehp.5919 available via *http://dx.doi.org/* [Online 9 January 2003]

Many chemicals have been released into the environment without prior evaluation of their endocrine activity at a molecular level, albeit the carcinogenic potential of these compounds is evaluated by routine mutagenicity testing. However, the concentration necessary to disrupt endocrine regulation may be lower than the carcinogenic level. Lifelong intake of even very low levels of these compounds may disturb the delicate hormone balance and compromise the reproductive fitness and health of many species. Likewise, the estrogenic potential of many environmental contaminants has been evaluated, but their impact on androgen-regulated parameters has traditionally been overlooked. The hormone sensitivity of the prostate and the rising incidence of prostate cancer identify it as a possible target organ for endocrine-disrupting chemicals.

Some environmental contaminants, such as hexachlorobenzene (HCB), have the potential to interfere with hormone regulation by mimicking or interfering with natural hormones. HCB is very persistent in the environment and impairs ovarian function in laboratory animals, decreases fertility, and decreases the weights of both seminal vesicles and ventral prostates (Elissalde and Clark 1979; Foster et al. 1996; Muller et al. 1978). In humans, the tissue samples of young boys with cryptorchidism (undescended testes) demonstrated elevated HCB levels compared with control surgical patients (Hosie et al. 2000). These observations of developmental abnormalities are consistent with those seen following disruption of hormone regulation.

Prostate cancer has become the most commonly diagnosed malignancy and the second leading cause of cancer-related death in North American men. However, other than its correlation to industrialization and the high incidence of prostate cancer in farmers, very little is known concerning the environmental factors that may facilitate the development of this disease or augment its progression (Hsing et al. 2000; Sharma-Wagner et al. 2000; Weston et al. 2000).

The steroid hormones responsible for development of the male reproductive tract are the androgens testosterone and dihydrotestosterone (DHT). In specific tissues, testosterone is reduced to the more potent, slower-dissociating androgen DHT (Zhou et al. 1995). DHT induces development of the prostate and male external genitalia by binding to the androgen receptor (AR). The AR is a member of the nuclear steroid receptor superfamily and is expressed in prostate cells and various tissues throughout the body. Upon androgen binding, it will translocate to the nucleus to form a homodimer, bind to androgen-responsive DNA elements, and initiate the transcription of androgen-regulated genes (Wong et al. 1995). The AR will bind a wide variety of ligand structures. In the case of antiandrogens, such as casodex, binding to the AR is thought to induce a receptor conformation that differs from that imposed by agonist binding, altering its ability to activate transcription (Eckert and Katzenellenbogen 1982; Hansen and Gorski 1986). Hormone antagonists may bind the receptor but prevent DNA binding and transcriptional activation, or they may promote receptor and DNA binding but nevertheless fail to initiate transcription (Truss and Beato 1993). Activation of the AR by various polyaromatic hydrocarbons has been shown to have detrimental effects on the development and function of the male reproductive system (Gray et al. 1999).

HCB is a known endocrine disruptor (Gocmen et al. 1989; Smith et al. 1987) that bioconcentrates in the fat of living organisms. HCB can persist in the environment for years, with an estimated half-life in soil of 23 years. It was used as a fungicide to protect onions, wheat seed, and other grains. Its production as an end point has been restricted in North America since 1971, but it is still formed as a major by-product in the manufacture of chemicals such as solvents, chlorine-containing compounds, and pesticides [Agency for Toxic Substances and Disease Registry (ATSDR) 1996]. U.S. Environmental Protection Agency studies have shown that detectable levels of HCB are found in the tissues of over 95% of the population (Robinson et al. 1990). Thousands of people were exposed to HCB in Kurdistan in eastern Turkey from 1955 to 1961. Retrospective analysis suggests an estimated intake of 2.6–4.1 mg/kg/day (Cripps et al. 1984; Gocmen et al. 1989).

In this article we demonstrate that HCB partially agonizes androgen action using a highly sensitive reporter gene assay. Cotransfection of the AR and an androgen-regulated luciferase reporter construct in a well-differentiated prostate cell line measured the ability of HCB to influence AR action. Results from a binding assay using thioredoxin-fused AR ligand binding domain demonstrated that HCB did not influence binding of androgen to its

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receptor. Our novel and highly sensitive *in vivo* approach used two strains of transgenic mice to test the impact of HCB on hormone action. The mE-RABP-CAT and LPB-CAT mouse express the chloramphenicol acetyl transferase (CAT) reporter gene under the direction of a highly androgen-sensitive promoter in the epididymis and prostate, respectively (Lareyre et al. 1999, 2000; Yan et al. 1997). HCB decreased CAT expression and prostate weights in both strains of mice.

Materials and Methods

Plasmids. The full-length rat AR cDNA cloned into pRc-CMV (Rennie et al. 1993), rat glucocorticoid receptor (GR) plasmid (Kasper et al. 1999), and human estrogen receptor (ER) (Smith et al. 1993) were used in the transfection-based assay. The luciferase reporter gene construct responsive to androgens and glucocorticoids (ARR3-luc) consisted of three rat probasin androgen-response elements upstream of the firefly luciferase vector (Snoek et al. 1996). The estrogen-responsive luciferase reporter gene construct (ERE-luc) plasmid consisted of a single vitellogenin estrogen response element cloned into a firefly luciferase vector (Portigal CL. Unpublished data). The pRL-tk vector obtained from Promega (Madison, WI, USA) contained a thymidine kinase promoter upstream of renilla luciferase cDNA. The pPSA-luc contains prostate-specific antigen (PSA) 5′-flanking DNA as described previously (Sato et al. 1997). All plasmid DNA was propagated in JM109 *Escherichia coli* and was prepared using a QIAGEN Maxiprep Kit (QIAGEN, Mississauga, Ontario, Canada).

Cell culture transfections. HCB (Aldrich Chemical Company, Milwaukee, WI, USA) was prepared as a 10-mM stock in ether. PC3 prostate cancer cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, Burlington, Ontario, Canada) supplemented with 5% dextran-coated charcoal-stripped fetal bovine serum. Plasmids were transiently transfected into the cells using Lipofectin Reagent (GibcoBRL). Each plate received 1.5 µg of the AR, ER, or GR plasmid, 1 µg of the corresponding reporter construct (ARR3-luc or ERE-luc), and 0.01 µg of pRL-tk. HCB and the appropriate hormone (2.5 nM DHT, 1 nM dexamethasone (DEX), or 1 nM estradiol) were added after a 6-hr incubation. Cells were harvested 24 hr later and lysed using Passive Lysis Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and the EG&G Berthold Microplate Luminometer LB 96V (Berthold Technologies, Bad Wildbad, Germany). The transfection experiments were also repeated in a similar manner using LNCaP prostate cancer cells cultured in RPMI 1640 defined medium (GibcoBRL). The cytotoxicity of HCB was assessed by staining samples with trypan blue and counting viable cells.

Cell culture ligand displacement assay. Recombinant rat thioredoxin-fused AR ligand binding domain (Trx-ARLBD) was obtained from PanVera (Madison, WI, USA) and diluted in binding buffer (50 mM Tris pH 7.5, 10% glycerol, 0.8 M NaCl, 1 mg/mL BSA, and 2 mM dithiothreitol). A stock of assay mix was prepared by combining 20 nM ^{3}H-R1881 (NEN Life Science Products, Inc., Boston, MA, USA) and 2% ethanol in binding buffer. A range of test compound concentrations was added to the diluted Trx-ARLBD and assay mix. After overnight incubation at 4°C, we added 33% hydroxylapatite (HAP) slurry [Fast Flow Hydroxylapatite (Calbiochem, San Diego, CA, USA) in 10 mM Tris pH 8.0 and 1 mM EDTA]. The HAP pellets were incubated on ice for 10 min, then washed three times with wash buffer (40 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA, and 1 mM EGTA). The HAP pellet was resuspended in ethanol and transferred to a scintillation vial. Scintillation counting was completed using the Beckman LS 6500 scintillation counter (Beckman, Mississauga, Ontario, Canada), and results were expressed as mean ± SEM.

In vivo *experiments.* All animal studies were conducted in accordance with the principles and procedures outlined by the Canadian Council on Animal Care and approved by institutional ethics committees. Animals were kept in standard conditions in 12-hr light:12-hr dark cycles in the animal care facilities in the Jack Bell Research Centre (Vancouver, British Columbia, Canada). The mice were fed a standard laboratory mouse chow and water *ad libitum*. The doses used in the experiment were chosen after a review of relevant literature (Cripps et al. 1984; Geyer et al. 1986; Gocmen et al. 1989). We tried to approximate human exposure while factoring in differences in the size, bioconcentration factors, and life span of mice. Bioconcentration factors (wet weight basis) of chemicals are between 3 and 47 times higher in humans than rats (Geyer et al. 1986).

The mice used in the first study were CD1 strain homozygous transgenic for an androgen-sensitive reporter gene. This consisted of a large section of the 5′-flanking sequence of the rat probasin gene linked to the bacterial CAT reporter gene (designated LPB-CAT). The transgene has been shown to be androgen sensitive and prostate specific (Yan et al. 1997). Five female mice were used in each of the five dose groups. All were fed 0.1 mL canola oil per day. The doses were as follows: control, canola oil alone; positive control, 0.1 mg/kg/day testosterone undecanoate in canola oil; low dose, 5 mg/kg/day HCB in canola oil; medium dose, 10 mg/kg/day; and high dose, 20 mg/kg/day HCB in canola oil. Females were dosed for a minimum of 1 month and then paired with males, one pair per cage. The exception is the testosterone-dosed dams, which could only be treated after conception; prepregnancy treatment with testosterone led to transient infertility. The dams were dosed throughout gestation (3 weeks) and lactation (3 weeks) until weaning. Male offspring were then treated for an additional 1 or 5 weeks until they reached 4 or 8 weeks of age, respectively.

In the second study we used a mouse strain designated mE-RABP-CAT. The epididymis of this transgenic mouse strain synthesizes a CAT reporter gene upstream of a retinoic acid-binding protein promoter (mE-RABP). The gene is specifically expressed in mouse mid/distal caput epididymis under the control of androgen (Lareyre et al. 1999, 2000). Seven females were dosed with 0.1 mL/day corn oil, and seven females were dosed with 10 mg/kg/day HCB in corn oil. The mice were paired and dosed as described above.

Treated male mice were sacrificed by cardiac exsanguination under methoxyfluorane anesthesia. Anogenital distance (AGD) and body weights were measured. The liver, left testis, left epididymis, and ventral prostate were removed, weighed and frozen. The ventral prostate and liver were first subdivided; half were frozen and the other half were fixed in

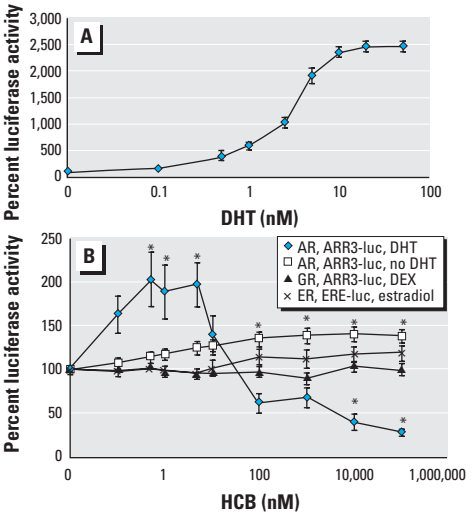

Figure 1. Impact of increasing compound levels on firefly luciferase production in PC3 cells. (*A*) Cells transfected with AR, ARR3-luc, and pRL-tk and incubated in the presence of DHT. (*B*) Cells transfected with the appropriate DNA constructs and incubated in the presence of half-maximal hormone levels and a range of HCB concentrations. An average of at least three experimental determinations, with three replicates each, were performed. Firefly luciferase values were normalized for transfection efficiency using activity of renilla luciferase as baseline levels. Data points indicate mean values; error bars indicate SEM.
*Significantly different from the control ($p < 0.01$).

10% neutral buffered formalin for histologic analysis. The right testis, right epididymis, and heart were also fixed for histologic examination. Serum testosterone and thyroxine (T_4) levels were tested. Blood from each animal was allowed to clot at room temperature for at least 15 min, and serum was obtained by centrifugation at 14,000 × *g* for 10 min. Serum was then immediately frozen at –20°C until it was examined for serum testosterone (Tumor Marker Lab, British Columbia Cancer Agency, Vancouver, British Columbia, Canada) and thyroid hormone levels (T_4 ELISA kit; Monobind, Costa Mesa, CA, USA). For the CAT assay, the organ of interest was homogenized and lysed. The protein level was measured using a Pierce BCA assay (Pierce Biotechnology, Rockford, Il, USA). One gram of protein in lysis buffer was heated to 65°C and added to a chloramphenicol, Tris, and ^{3}H-acetyl coenzyme A (CoA) solution. ScintiLene scintillation fluid (Fisher Scientific, Nepean, Ontario, Canada) was layered on top, and then scintillation counts were taken over time. The slope of disintegrations per minute over time corresponds to CAT activity.

Histopathologic analysis. Slides containing sections of prostate, liver, heart, or epididymis were stained with hematoxylin-eosin. The same pathologist examined each section in a randomized manner using a photonic microscope.

Statistical analysis. All data sets were tested for uniform distribution. Statistical significance (p = 0.05) among the various parameters assessed was established by a Student's *t*-test when a single treatment was compared with the control or by analysis of variance when the comparison was between several groups. Upon demonstration of statistical significance, Dunnett's multiple comparison test indicated which groups were significantly different from the control group. Statistical analyses were performed using JMP statistical software (SAS Institute, Cary, NC, USA).

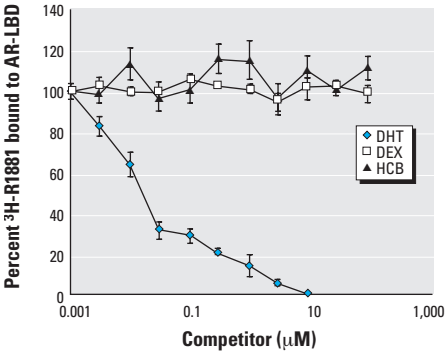

Figure 2. Effects of nontritiated potential ligand added to thioredoxin-fused AR ligand binding domain (Trx-AR-LBD). Trx-AR-LBD aliquots were incubated in the presence of 20 nM ^{3}H-R1881, and then DHT or HCB or DEX was added. DHT displaced R1881 and bound the AR, whereas DEX and HCB did not.

Results

***Hexachlorobenzene modulates androgen activity* in vitro.** Based on the observation that HCB is highly persistent and has the potential to interfere with hormone regulation, we wanted to test if HCB would mimic or inhibit steroid hormones important in the prostate using a tissue cell-culture–based assay. Our approach used DNA constructs containing hormone-responsive binding sites connected to a firefly luciferase reporter gene. The amount of luciferase produced was proportional to the degree of transcriptional activity induced by the ligand-bound receptor. The pRL-tk was cotransfected as a transfection control and was constitutively expressed.

Increasing concentrations of DHT, a positive control, induced rising firefly luciferase production in PC3 cells transfected with the ARR3-luc reporter plasmid and the full-length rat AR (Figure 1A). After this standardization of our assay, we asessed the impact of HCB. In the presence of half-maximal DHT, low levels of HCB enhanced the androgen-responsive transcription of the luciferase reporter gene up to 2-fold higher than DHT alone. High levels of HCB suppressed this androgen-mediated activity (Figure 1B). This HCB-induced modification of transcriptional activity was dependent upon the presence of the AR (data not shown), and it appeared to be specific to the AR. In the presence of GR or ER and their respective hormones, HCB did not interfere with hormone-inducible transcription (Figure 1B). These experiments were repeated in two different prostate cell lines, PC3 and LNCaP, with different promoters (ERE-luc, ARR3-luc, and PSA-luc). The same trend was seen in both cell lines regardless of whether PSA-luc or ARR3-luc was used. In LNCaP cells, HCB will act to agonize androgen action in the presence of the endogenous mutated AR, but this impact is insignificant when compared with the response elicited by the transfected receptors. Each set of test conditions was reproduced in triplicate, and each experiment was repeated a minimum of three times. Based on our observations that HCB impacts *in vitro* AR action specifically, the next step was to determine if HCB binds directly to the AR.

***Hexachlorobenzene was not an AR ligand* in vitro.** Tritiated R1881, a synthetic androgen, was added to AR ligand binding domain. Nonradioactive ligand (DHT, DEX, or HCB) was added and displaced the tritiated R1881 if the affinity was greater. The pellets were washed and assessed for levels of radioactivity. If binding to the AR occurred, there was a decrease in scintillation counts. Results from this assay indicated that the DHT control was a ligand of the AR, but HCB and DEX were not (Figure 2).

***Hexachlorobenzene alters androgen-responsive parameters* in vivo.** Male mice were treated throughout gestation and lactation via maternal exposure. Three weeks after the date of birth, the male offspring were weaned and fed individually for an additional 1 or 5 weeks to correspond to a sacrifice age of 4 or 8 weeks, respectively. Two different sacrifice points were used because some effects such as delayed or precocious puberty are seen at the immature/prepubescent time point of 4 weeks of age. Other effects can only be observed when the animal is mature, such as abnormal development, or upon sufficient accumulation of the chemical to produce detectable effects.

High-dose HCB decreased CAT activity in adult LPB-CAT male mice. CAT reporter activity in the LPB-CAT mice corresponded to the degree of androgen activity in the prostate (Figure 3). In the 4-week-old mice, the androgen-responsive CAT activity of the testosterone-dosed mice was significantly higher (p < 0.01). In the 8-week-old mice, the CAT activity of mice treated with the medium and high HCB dose was significantly (p < 0.05) lower. We used a second strain of mice to confirm that the observations were not unique to the LPB-CAT mice. mE-RABP-CAT mice also expressed an androgen-responsive CAT reporter. However, several differences exist: the promoter was based on a fragment from the epididymal retinoic acid-binding protein rather than the probasin gene, the transgene was expressed specifically in the epididymis rather than the prostate, and these mice have a C57BL/6 rather than a CD1 background. No significant change in CAT activity was observed between the 10 mg/kg/day HCB-treated and control mice at 4 weeks of age, but there was a significant (p =0.05) decrease in androgen-responsive epididymis-specific CAT activity of 8-week-old mice dosed with 10 mg/kg/day HCB compared with controls (Figure 4). Thus, it was demonstrated that HCB could impact androgen-regulated transcriptional activity in both strains of mice.

The weight of the prostate is also a measure of androgen activity. The prostate is responsive to androgen levels, as treatment with antiandrogens or castration will decrease the prostate weight (Iguer-Ouada and Verstegen 1997; Schroder 1994). Administration of exogenous testosterone to immature males accelerates prostatic growth so that maximal prostatic size is achieved precociously (Cunha et al. 1987). Prostate development is induced by DHT, and although we used an oral form of testosterone (testosterone undecanoate), its conversion to DHT has been reported (Horst and Erdmann 1980). The average prostate weight of 4-week-old LPB-CAT mice treated with either testosterone or the low dose of HCB was significantly higher than the average for the controls (Figure 5). In the 8-week-old LPB-CAT mice, treatment with the high dose of HCB significantly lowered the average

prostate weight compared with the control average ($p < 0.01$). A decrease in prostate weight implied a delay in sexual maturity and was previously observed following exposure to vinclozolin (Gray et al. 1994), genistein (Delclos et al. 2001), or soy phytoestrogens (Weber et al. 2001). Prostatic acini convert from a nondilated to a dilated form around the time of puberty and could therefore be used as a marker of sexual maturity. Exposure to the low dose of HCB in 4-week-old mice caused a significant increase in the percentage of cases where dilated prostate acini are observed. However, dilated prostatic acini were not observed in any of the high HCB dose samples. Dilated acini were observed in every 8-week-old mouse prostate (Figure 6). These data suggest that HCB agonized androgen action at low doses, but antagonized it at high concentrations.

Other androgen-sensitive organs include the epididymis and the testis; AGD is also affected by androgen. Treatment with testosterone, low-dose HCB, and medium-dose HCB in the 4-week-old (Table 1) and low-dose HCB in the 8-week-old LPB-CAT (Table 2) males induced a higher average epididymis weight. Thus, low-dose HCB treatment increased the weight of the epididymis, an androgen-sensitive organ, at both time points. Exposure to testosterone propionate (Orgebin-Crist et al. 1983) increases the weight of the epididymis, whereas ethinylestradiol (Kinomoto et al. 2000) or flutamide (Toyoda et al. 2000) exposure decreases it. The AGD is a developmental marker and is larger in males than females. In this study, the medium dose of HCB significantly increased the average AGD compared with the control group (Table 2). Exposure to antiandrogens has been shown to decrease the AGD (Gray et al. 2001; Hib and Ponzio 1995; McIntyre et al. 2001). In 4-week-old LPB-CAT mice (Table 1), average testis weights of the mice treated with testosterone or the low-dose HCB were significantly higher than the control ($p < 0.05$). In the current study, the impact of HCB on the testes was not as pronounced as other organs of the male reproductive tract. This may have been because the mammalian testis accumulates lower levels of organochlorine chemicals compared with other tissues and affords the germ cells some protection from the potentially toxic compounds (Cooke et al. 2001).

The serum testosterone levels of both the 4-week-old and 8-week-old LPB-CAT mice were measured by the British Columbia Cancer Agency Tumor Marker Lab; because of the temporal nature of hormone release, the values were highly variable. No statistically significant changes were observed, but the average testosterone level of the 4-week-old testosterone-dosed mice was about 3–4 times higher than the control average (data not shown). In a previous study, mice dosed with 250 mg HCB/kg for 21 days demonstrated an increase *in vitro* metabolism of [^{3}H] testosterone, a decrease in serum concentrations of testosterone, and a decrease in weights of seminal vesicles and ventral prostates (Elissalde and Clark 1979).

The T_4 thyroid hormone levels of 4- and 8-week-old mice were tested, and no visible or statistically significant changes or trends were observed (data not shown). Thyroid hormone levels were tested, as previous studies showed that HCB induced hypothyroidism, albeit at higher doses (Foster et al. 1993; van Raaij et al. 1994).

Acute toxicity was not observed. Chronic ingestion of HCB is associated with hepatomegaly, loss of body weight, wasting of skeletal muscles, leukocytosis, and enlarged thyroid (Hayes and Laws 1991). We measured heart, liver, and body weight to assess signs of toxicity in the treated mice. In this study, we studied endocrine-specific effects and attempted to keep the exposure levels below those that are overly toxic. Loss of body weight was not observed in test mice upon exposure to HCB. The average body weight was significantly ($p < 0.01$) higher in the groups treated with the low and medium doses of HCB (Tables 1 and 2). The average liver weights significantly increased upon exposure to HCB, but lesions were not observed (Tables 1 and 2). The increase in liver weight may have been due to altered glycogen storage, as there was a dose-dependent increase in centrilobular hepatocellular vacuolization (data not shown). Multifocal hepatic necrosis and mild inflammation of periportal spaces were observed in a few of the samples (data not shown). These alterations are common and were not associated with a specific treatment group. Therefore, although the livers of the high-dose HCB group were enlarged and

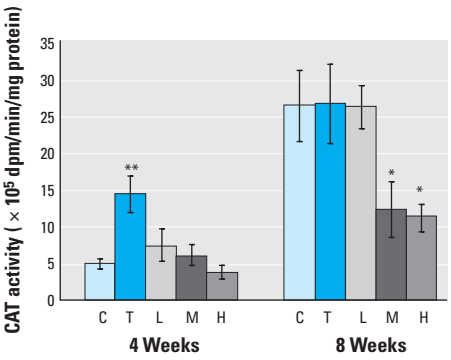

Figure 3. Androgen-responsive CAT activity in LPB-CAT mice increased in 4-week-old mice (prepubescent) treated with testosterone and decreased in 8-week-old mice (sexually mature) treated with HCB. Abbreviations: C, control; H, high-dose HCB; L, low-dose HCB; M, medium-dose HCB; T, testosterone.
*Significantly different from the control ($p < 0.05$).
**Significantly different from the control ($p < 0.01$).

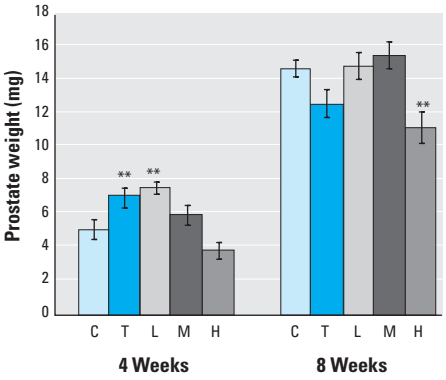

Figure 5. Effects of HCB or testosterone in LPB-CAT mice dosed until sacrifice at 4 weeks of age (prepuberty) or 8 weeks of age (sexually mature). Abbreviations: C, control; H, high-dose HCB; L, low-dose HCB; M, medium-dose HCB; T, testosterone. The androgen-sensitive prostate weight increased in the low-dose HCB-treated immature mice but decreased in response to the high HCB dose in sexually mature animals.
**Significantly different from the control ($p < 0.01$).

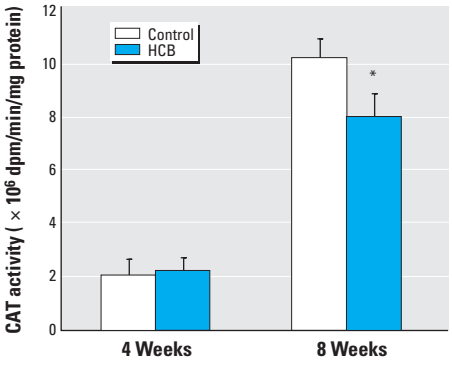

Figure 4. Effects of HCB in mE-RABP-CAT mice dosed until sacrifice at 4 weeks of age (prepubescent) or 8 weeks of age (sexually mature). There was a significant decrease in CAT activity at 8 weeks of age in response to treatment with 10 mg/kg/day compared with controls dosed with vehicle alone.
*Significantly different from the control ($p = 0.05$).

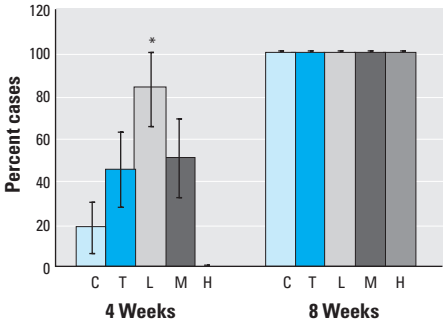

Figure 6. Percentage of cases in which the presence of dilated acini was observed in the prostates of LPB-CAT mice at 4 weeks of age (prepubescent) and 8 weeks of age (sexually mature). Abbreviations: C, control; H, high-dose HCB; L, low-dose HCB; M, medium-dose HCB; T, testosterone. Progression from nondilated to dilated acini was a marker for sexual maturity.
*Significantly different from the control ($p < 0.05$).

slightly altered glycogen storage was observed, no severe liver toxicity was linked with the doses of HCB used. Heart weight was increased in the highest HCB dose group (Tables 1 and 2). The hearts examined had a normal histological architecture and no lesion was detected in the sections (data not shown).

Discussion

The endocrine system acts as a communication network, and the signals are transmitted via hormones. The levels of individual hormones are tightly controlled for effective endocrine regulation and production of the appropriate biological response at the desired time. Endocrine disruptors are compounds that are able to mimic or block this natural biological process. Because of a certain degree of promiscuity of steroid hormone receptors, they are able to bind a variety of distinct molecules, albeit with different binding affinities. The endocrine and reproductive effects of environmental contaminants are believed to result from *a*) mimicking endogenous hormones such as estrogens and androgens, *b*) antagonizing normal endogenous hormones, *c*) altering the pattern of synthesis and metabolism of natural hormones, *d*) modifying hormone receptor levels, or *e*) interfering with steroid-binding protein or steroid transport. Many of the endocrine-disruptor studies have traditionally focused on the impact of environmental estrogens. Novel data have emerged that demonstrate the impact of endocrine disruptors on the androgen axis.

Vinclozolin and *p,p′*-DDE [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethylene] act as AR ligands and antagonize transcriptional activity *in vitro*. Exposure to *p,p′*-DDE and vinclozolin causes malformations of the male reproductive tract in neonates, including a decrease in prostate weights, suppression of androgen-regulated genes, reduced AGD, retained nipples, and reduced ventral prostate weights (Gray et al. 1994; Kelce et al. 1997). Although it is not an AR ligand, similar results are seen upon exposure to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (Mably et al. 1992). Compounds such as HCB and TCDD may influence androgen action via the aryl hydrocarbon receptor (AhR). The AhR is expressed in prostate epithelial cells, and stimulation of the AhR by various polyaromatic hydrocarbons has detrimental effects on the male reproductive system. The AR is also expressed in prostate cells, and crosstalk between the AR and AhR has been shown *in vitro*. TCDD is the most potent inducer of AhR activity. Testosterone inhibited TCDD-induced CYP1A1 activity in a dose-dependent manner. Reciprocally, testosterone-dependent transcriptional activity and testosterone-regulated PSA expression in the prostate cell line LNCaP was inhibited by TCDD (Jana et al. 1999). Ligand-independent activation of the AR via the estrogen-induced growth factor pathway has also been demonstrated in prostate organ culture (Gupta 2000). In some circumstances, crosstalk may be a normal part of signaling in the prostate, but inappropriate activation or suppression will disrupt the function of the cell and organ development.

HCB was introduced in 1945 as a fungicide that interferes with amine and thiol metabolism to slow the growth rate and sporulation of fungi. It is still formed as a major by-product and contaminant in the manufacture of chemicals such as solvents, chlorine-containing compounds, and pesticides (ATSDR 1996). Total daily intake from air, food, and soil by adults in the general North American population is about 3 ng/kg/day (Newhook and Meek 1994). Average intake of HCB by breast-feeding infants in the general population is much higher (Uhnak and Szokolay 1983), and lactational transfer of HCB has been demonstrated (Nakashima et al. 1997). Childhood exposure to HCB may influence reproduction or physical and mental development. A previous study demonstrated elevated levels of HCB in young males with undescended testes (Hosie et al. 2000). HCB has a potent capacity for accumulation from the environment into the fatty tissues of living organisms (Ernst 1986; Hayes and Laws 1991). Many of the environmental contaminants are lipophilic, and androgens have been shown to stimulate the accumulation of lipids in LNCaP prostate cancer cells (Swinnen et al. 1996). Environmental contaminants have been implicated in the rising incidence of prostate cancer, and insight into the mechanisms of endocrine disruption will help to clarify their role.

In this study, mice were orally dosed with HCB levels similar to highly exposed individuals, but elevated compared with average human exposure levels. At low doses, HCB acted as an androgen agonist *in vitro* and *in vivo*. It increased the production of the androgen responsive reporters, firefly luciferase in PC3 cells, and the presence of dilated acini in the prostates of sexually immature LPB-CAT mice. However, higher doses of HCB apparently antagonized androgen action, as the production of firefly luciferase was reduced. HCB

Table 1. Mean (SEM) organ weights and AGD of 4-week-old LPB-CAT mice treated with canola oil, testosterone, or HCB.

Organ	Control ($n = 11$)	Testosterone ($n = 9$)	Low HCB ($n = 6$)	Medium HCB ($n = 8$)	High HCB ($n = 5$)
Prostate (g)	0.0051	0.0071**	0.0077**	0.0060	0.0038
	(0.0006)	(0.0005)	(0.0004)	(0.0006)	(0.0005)
Testis (g)	0.0596	0.0700*	0.0702*	0.0645	0.0600
	(0.0012)	(0.0036)	(0.0034)	(0.0023)	(0.0041)
Epididymis (g)	0.0114	0.0151**	0.0145**	0.0134**	0.0106
	(0.0005)	(0.0003)	(0.0002)	(0.0005)	(0.0011)
AGD (mm)	8.43	9.11	8.53	9.51	9.17
	(0.25)	(0.35)	(0.52)	(0.36)	(0.25)
Body weight (g)	20.45	23.65**	25.81**	23.90**	20.31
	(0.60)	(0.80)	(0.71)	(0.49)	(1.06)
Liver (g)	1.037	1.297**	1.257**	1.299**	1.222**
	(0.046)	(0.024)	(0.072)	(0.035)	(0.060)
Heart (g)	0.105	0.132**	0.136**	0.131**	0.122**
	(0.004)	(0.005)	(0.004)	(0.003)	(0.006)

*Significantly different from the control ($p < 0.05$). **Significantly different from the control ($p < 0.01$).

Table 2. Mean (SEM) organ weights and AGD of 8-week-old LPB-CAT mice treated with canola oil, testosterone, or HCB.

Organ	Control ($n = 10$)	Testosterone ($n = 9$)	Low HCB ($n = 6$)	Medium HCB ($n = 8$)	High HCB ($n = 5$)
Prostate (g)	0.0148	0.0127	0.0150	0.0156	0.0112**
	(0.0006)	(0.0008)	(0.0009)	(0.0009)	(0.0010)
Testis (g)	0.1271	0.1339	0.1342	0.1241	0.1264
	(0.0033)	(0.0046)	(0.0032)	(0.0083)	(0.0051)
Epididymis (g)	0.0276	0.0306	0.0323*	0.0280	0.0268
	(0.0009)	(0.0012)	(0.0009)	(0.0011)	(0.0020)
AGD (mm)	12.17	11.54	11.21	13.45**	11.90
	(0.55)	(0.30)	(0.26)	(0.22)	(0.72)
Body weight (g)	27.99	29.43	32.72**	31.89**	29.12
	(0.79)	(0.67)	(0.98)	(0.50)	(0.97)
Liver (g)	1.327	1.482	1.549	1.733**	1.848**
	(0.043)	(0.069)	(0.073)	(0.062)	(0.070)
Heart (g)	0.148	0.162	0.168*	0.162	0.172*
	(0.004)	(0.006)	(0.005)	(0.006)	(0.009)

*Significantly different from the control ($p < 0.05$). **Significantly different from the control ($p < 0.01$).

did bind directly to the AR and may influence androgen action by altering steroid transport or via receptor crosstalk. In LPB-CAT mice, the high dose of HCB downregulated CAT activity in 8-week-old mice, decreased the presence of dilated prostatic acini, and lowered the average prostate weight. In mE-RABP-CAT mice, HCB decreased the CAT activity of 8-week-old mice.

These data provide conclusive evidence of HCB acting as an endocrine disruptor in mice and demonstrates its potential to impact the human androgen axis. HCB can interfere with the transcriptional activity of androgen-regulated genes and the downstream effects, thus amplifying its potential endocrine-disrupting impact. The fact that HCB may affect the androgen-signaling pathway in a different manner depending on the dose should reinforce the concept that environmental xenobiotics, though present at low doses, may pose a threat to human health.

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