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Several Synthetic Chemicals Inhibit Progesterone Receptor-Mediated Transactivation in Yeast

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The human progesterone receptor (hPR) B-form and a progesterone-sensitive reporter were expressed in yeast and used to screen a library of synthetic chemicals for their ability to function as agonists or antagonists of hPR. The transcriptional activity of hPR was not increased in the presence of over 40 individual chemicals. Seven chemicals decreased progesterone-dependent activity in yeast. The most effective chemicals were 6-hydroxychrysene, 1-hydroxypyrene, 4-hydroxy, 2′,4′,6′-trichloro biphenyl, and 4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl. The decrease of progesterone-mediated transactivation strongly correlated with their displacement of [^{3}H]progesterone from hPR. The absence of the hydroxyl group on the above chemicals completely abolished their inhibitory activity. The other chemicals which decreased progesterone activity were endosulfan II, endosulfan sulfate, and lindane. These chemicals did not inhibit [^{3}H]-progesterone binding, suggesting that they inhibit progesterone action by interacting with a region of hPR distinct from binding [^{3}H]progesterone or by a mechanism independent of hPR. These results highlight the utility of yeast for screening hormonally-active chemicals. In addition, hydroxylation appears to be essential for the interaction of some chemicals with hPR.

We have proposed environmental signaling as a new field within toxicology (1). Environmental signaling involves the functional interaction of exogenous chemicals with endogenous systems, such as steroid hormone receptors in the endocrine system. The best-studied example is the concept of environmental estrogens and the discovery and characterization of several environmental chemicals that bind and activate the estrogen receptor (ER) *in vitro* and *in vivo* (2-4). Recently, synthetic chemicals have been shown to bind and interact with the androgen (5) and retinoid receptors (6). These findings suggest the possibility that exogenous chemicals may interact with numerous hormone receptors.

A well-studied example of environmental estrogens are the hydroxylated polychlorinated biphenyls 4-hydroxy, 2′,4′,6′-trichloro biphenyl and 4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl. These chemicals increased uterine weight in treated mice (7) and sex-reversed male determined turtle embryos (8), both estrogen-dependent processes. The hydroxylated PCBs interact with ER *in vitro* suggesting a mechanism for their effects (7, 9). Another class of chemicals that have been described as estrogenic are the pesticides endosulfan, toxaphene and dieldrin (2). These chemicals increased growth of estrogen-sensitive MCF-7 cells (2) and in combination with certain chemicals can produce estrogenic responses in a synergistic manner (9). The difficulty in determining which chemicals are estrogenic has been a lack of conserved structural features. This observation has prompted the need for analyzing the functional interaction of chemicals with specific hormone receptors (10). Previously, we have utilized ER and estrogen-sensitive reporter expressed in yeast to characterize the (anti)-estrogenic activities of various natural and synthetic chemicals (9, 11-15). Several chemicals have been shown to be active in yeast expressing ER indicating that this *in vitro* assay might be applicable for other steroid hormone receptors.

In this report, we have expressed the hPR B-form and a progesterone-dependent reporter in yeast. Using

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Abbreviations: DMSO, dimethylsulfoxide; ER, estrogen receptor; hPR, human progesterone receptor; ONPG, *o*-nitrophenyl-*β*-D-galactopyranoside; PRE, progesterone response element.

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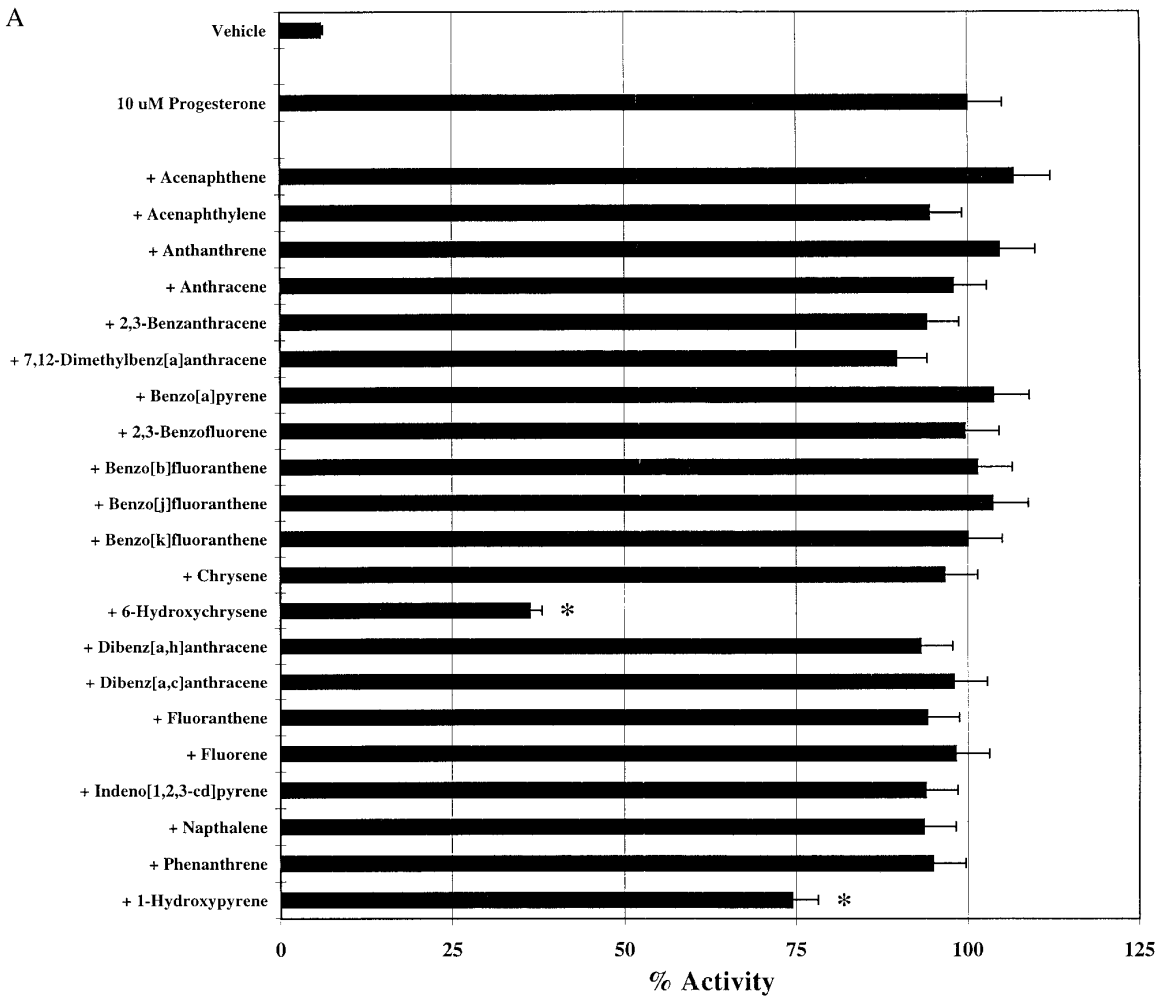

FIG. 1. The inhibition of PR activity with various chemicals in yeast strain hPR-PRE. Strain hPR-PRE was incubated with vehicle, 10 nM progesterone in the presence or absence of various chemicals at 1 μM, and grown for 12 h, and then β-galactosidase activity was measured (A & B). The % activity is defined as a percentage of β-galactosidase activity of progesterone in the absence of competitor. The * indicates % activity that is significantly different from 10 nM progesterone (10 nM progesterone produced 250 Miller units).

this system we have screened a library of synthetic chemicals for their ability to function as hPR agonists or antagonists. None of the chemicals tested increased hPR-mediated transactivation. Seven chemicals decreased progesterone-dependent activity in yeast. Some chemicals appear to strongly bind hPR whereas other chemicals appear to exert their effects through a different mechanism(s).

MATERIALS AND METHODS

Materials. Progesterone and the amino acids for culturing yeast were purchased from Sigma Chemical Co. (St. Louis, MO.). [^{3}H]-progesterone (85 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE). All the chemicals at the indicated purities were purchased from AccuStandard Inc. (New Haven, CT.). 4-hydroxy, 2′,4′,6′-trichloro biphenyl and 4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl were custom synthesized from AccuStandard Inc. (New Haven, CT.).

Yeast assays. Yeast strains hPR-PRE was created by transforming strain DY150 (MATa, ura 3-1, leu 2-3, 112 trp 1-1, his 3-11, 15 ade 2-1, can 1-100) with YEPhPR-B, an expression plasmid for the B form of hPR, and YEPEG2LEU2, a *lacZ* reporter containing 2 progesterone response elements (PRE) (12). Strain hPR-PRE was grown overnight at 30 °C in synthetic medium-tryptophan, leucine. The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with dimethylsulfoxide (DMSO), progesterone, the various synthetic chemicals or progesterone in the presence of the chemicals. Progesterone was prepared in DMSO and the concentration of DMSO in the assay was 0.1%. The same volume of solvent was used for each concentration of the various chemicals and progesterone tested. None of the chemicals inhibited the growth of the yeast at the concentrations tested.

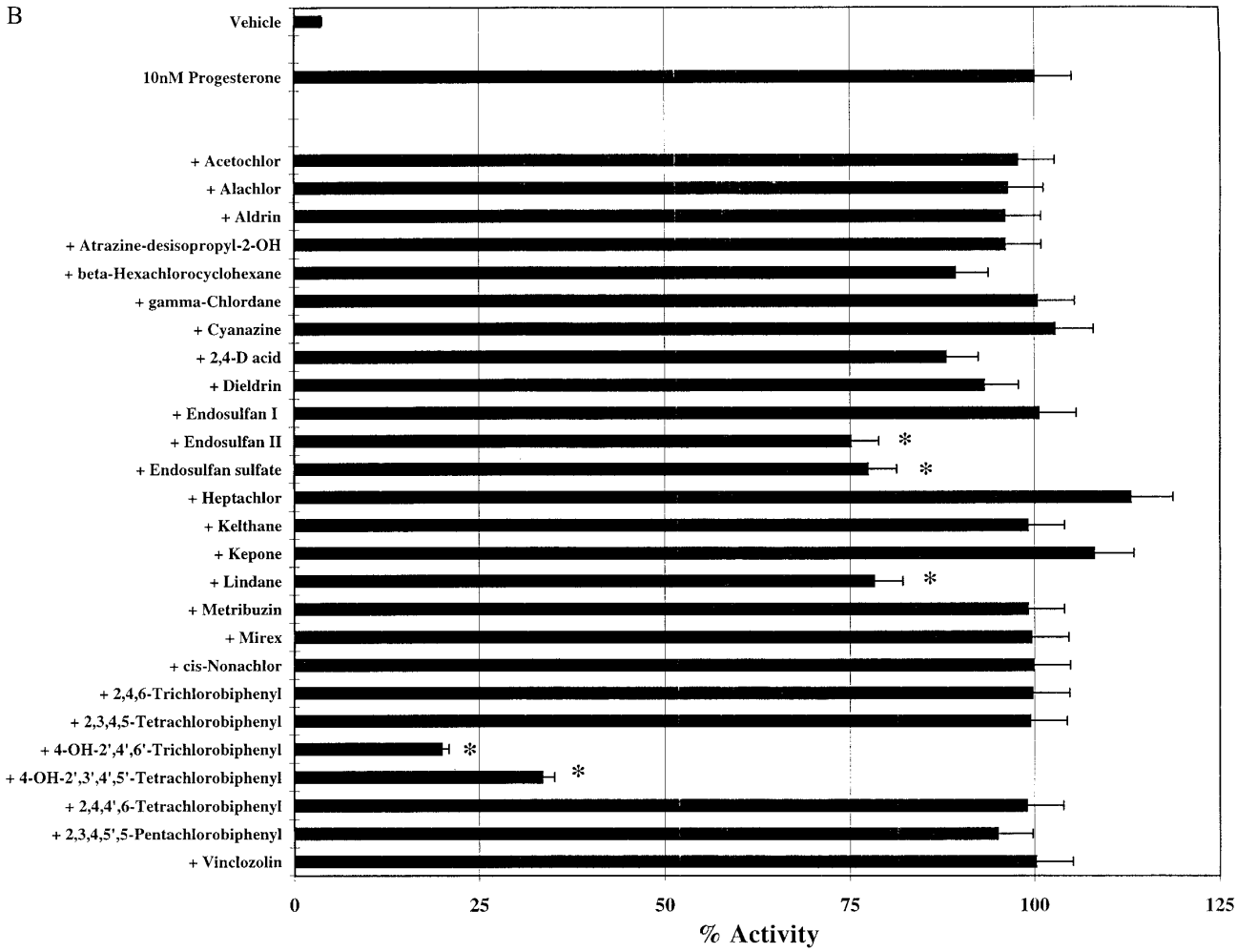

FIG. 1—*Continued*

β-Galactosidase assays. The yeast cells were collected by centrifugation, resuspended in 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol) and permeabilized by the addition of 6 μl of $CHCl_3$ and 4 μl 0.1% SDS followed by vortexing for 25 s. The reactions were equilibrated at 30 °C for 10 min, then 160 μl of ONPG (4 mg/ml in Z-buffer) was added and the reactions returned to 30 °C for between 5 and 60 min. The reactions were terminated by the addition of 400 μl 1M $NaCO_3$, the cell debris removed by centrifugation and the absorbance at 420 nM measured (A_{420}). The growth of the yeast strains was monitored by measuring the absorbance at 600 nM (A_{600}). Miller units were determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells $\times$ volume of culture $\times$ length of incubation)] $\times$ 1000. Statistics were performed by one-way ANOVA least significant difference test (Microsoft Excel). Significant differences were defined when $p<0.05$. The data are representative of three independent experiments with three replicates.

Whole cell binding assays. Strain hPR-PRE was grown overnight at 30 °C in synthetic medium-tryptophan, leucine. The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with 2 nM [^{3}H]progesterone in the presence or absence of various concentrations of radioinert progesterone or the various chemicals. None of the chemicals inhibited the growth of the yeast at the concentrations tested. The cells were collected by centrifugation, washed three times with ice-cold PBS, suspended in PBS and the radioactivity measured in a liquid scintillation counter. The data are representative of three independent experiments with three replicates.

RESULTS AND DISCUSSION

To examine the functional interaction of various synthetic chemicals with the hPR, strain hPR-PRE was created by expressing hPR and a progesterone-sensitive reporter in yeast (12). To screen a library of synthetic chemicals for activity as hPR agonists, yeast hPR-PRE was incubated with 1 μM of the chemicals. None of the chemicals tested induced β-galactosidase activity in yeast (data not shown; see Fig. 2 for list of chemicals tested).

The possibility that some chemicals in the library

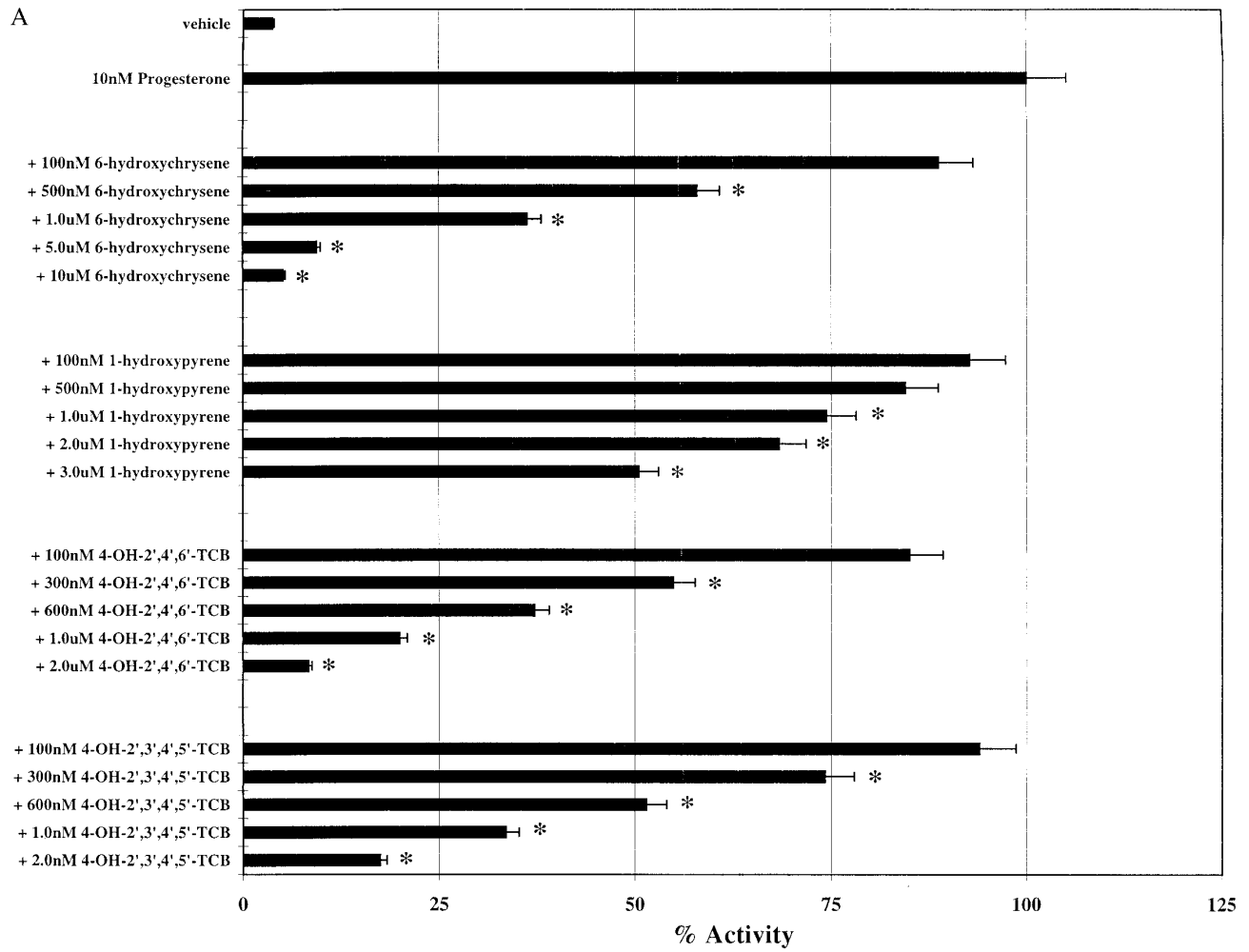

FIG. 2. Dose-response curve of several chemicals in the presence of progesterone in yeast strain hPR-PRE. Strains hPR-PRE were incubated with vehicle, 10 nM progesterone in the presence or absence of the indicated chemicals at varying concentrations (A & B). The % activity is defined as a percentage of β-galactosidase activity of progesterone in the absence of competitor. The * indicates % activity that is significantly different from 10 nM progesterone. 4-OH-2′,3′,4′-TCB and 4-OH-2′,3′,4′,5′-TCB represent 4-hydroxy 2′,4′,6′-trichloro biphenyl and 4-hydroxy 2′,3′,4′,5′-tetrachloro biphenyl, respectively.

functioned as antagonists of hPR was evaluated by coincubation of strain hPR-PRE with 10 nM progesterone (the concentration of progesterone that induced maximal β-galactosidase activity) (12) and 1 μM of the various chemicals. The chemicals which significantly reduced β-galactosidase activity in yeast were 6-hydroxychrysene, 1-hydroxypyrene, 4-hydroxy 2′,4′,6′-trichloro biphenyl and 4-hydroxy 2′,3′,4′,5′-tetrachloro biphenyl, endosulfan II, endosulfan sulfate and lindane (Fig. 1A and B). Next, a dose-response curve with these chemicals in the presence of progesterone was performed in yeast. 4-hydroxy 2′,4′,6′-trichloro biphenyl was the most effective inhibitor of progesterone activity with an IC_{50} (the concentration required for a 50% reduction in β-galactosidase activity) of approximately 400 nM (Fig. 2A). Both 4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl and 6-hydroxychrysene had an IC_{50} value of 600 nM (Fig. 2A). Endosulfan II, 1-hydroxypyrene, endosulfan sulfate and lindane had IC_{50} values of 2.5, 3, 5 and 7.5 μM, respectively (Fig. 2A and B).

The inhibition of hPR activity in yeast was further tested by performing competition binding assays in yeast with [^{3}H]progesterone. Yeast were incubated with 2 nM [^{3}H]progesterone in the presence or absence of various concentrations of radioinert progesterone or the chemicals tested in Fig. 2 and the resulting [^{3}H]-progesterone-hPR complexes measured. A 300-fold excess of progesterone decreased [^{3}H]R5020 binding to hPR by 70% (Fig. 3A). The most effective competitor for

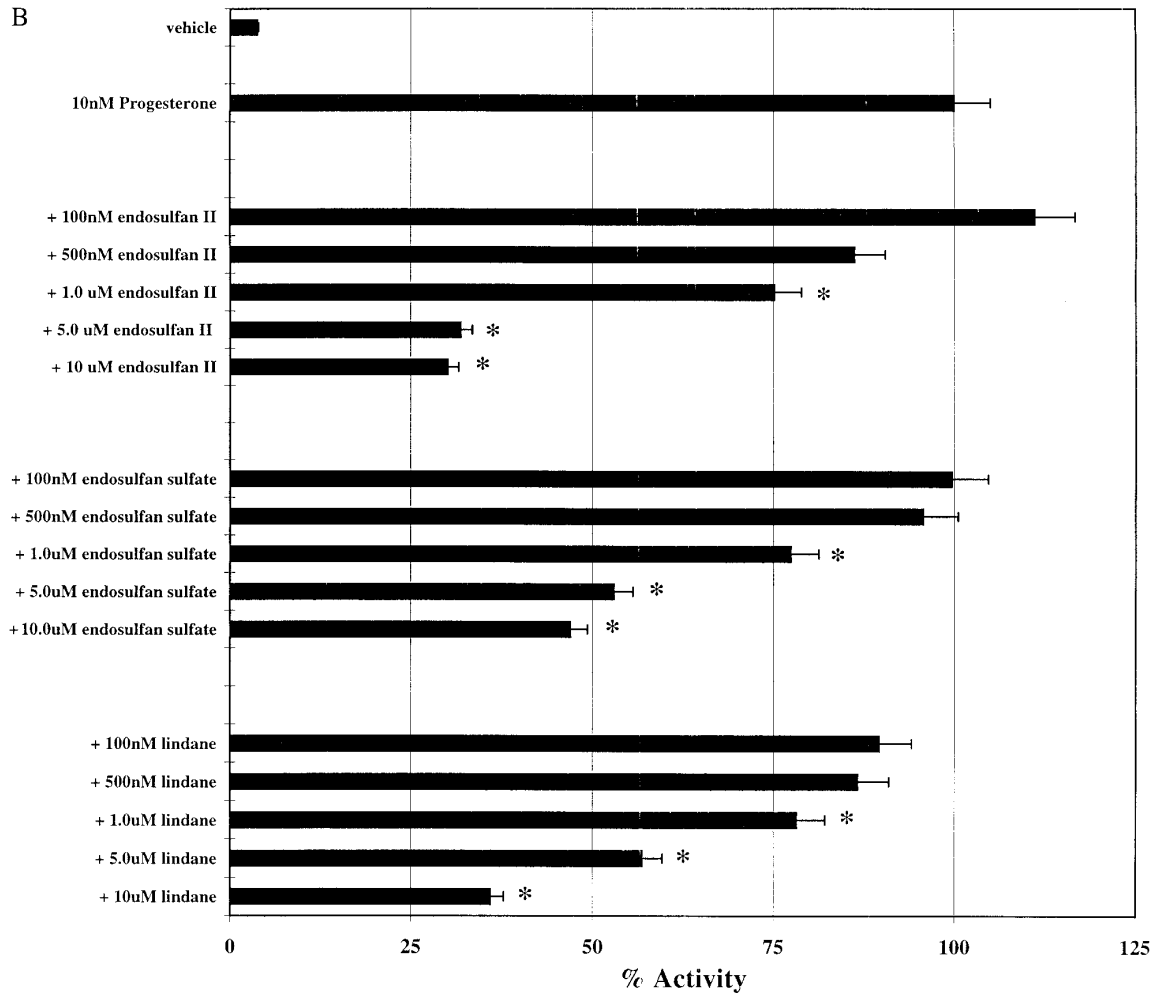

FIG. 2—*Continued*

[^{3}H]progesterone-binding was 6-hydroxychrysene with an IC_{50} value (the concentration of chemical required to reduce binding by 50%) of 1 μM (Fig. 3A). 4-hydroxy 2′,4′,6′-trichloro biphenyl, 4-hydroxy 2′,3′,4′,5′-tetrachloro biphenyl and 1-hydroxypyrene had IC_{50} values of 1.5, 2.5 and 3 μM, respectively. Endosulfan II, endosulfan sulfate and lindane did not significantly reduce [^{3}H]progesterone (Fig. 3B).

Numerous reports have demonstrated that PR reduces ER-mediated transactivation (16, 17). This observation has been suggested to be an underlying molecular mechanism for the opposing effects of estradiol and progesterone in the female reproductive tract. The strong estrogenic activity of 4-hydroxy 2′,4′,6′-trichloro biphenyl and 4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl in mice (8) and turtles (9) suggests that these compounds may not only exert activity through ER but also prevent repression of ER activity by PR. In some cases, the cross-talk of environmental chemicals with multiple hormone receptors may enhance a specific hormonal effect.

The role of hydroxylation of some synthetic chemicals as a requirement for the interaction and inhibitory activity with hPR is highlighted in this study. 6-hydroxychrysene, but not chrysene, and 1-hydroxypryene decreased progesterone-dependent activity. Furthermore, the hydroxylated PCBs 4-hydroxy 2′,4′,6′-trichloro biphenyl and 4-hydroxy 2′,3′,4′,5′-tetrachloro biphenyl were the most potent chemicals for inhibiting the activity of progesterone in yeast. PCBs lacking a hydroxyl group (2,4,6-trichloro biphenyl and 2,3,4,5-tetrachloro biphenyl) or containing a chlorine substitution for the hydroxyl group (2,4,4′,6-tetrachloro biphenyl and 2,3,4,4′,5-pentachloro biphenyl) were inactive. 4-hydroxy 2′,4′,6′-trichloro biphenyl, 4-hydroxy 2′,3′,4′,5′-tetrachloro biphenyl, 6-hydroxychrysene and 1-hydroxypryene strongly displaced [^{3}H]progesterone from hPR indicating that

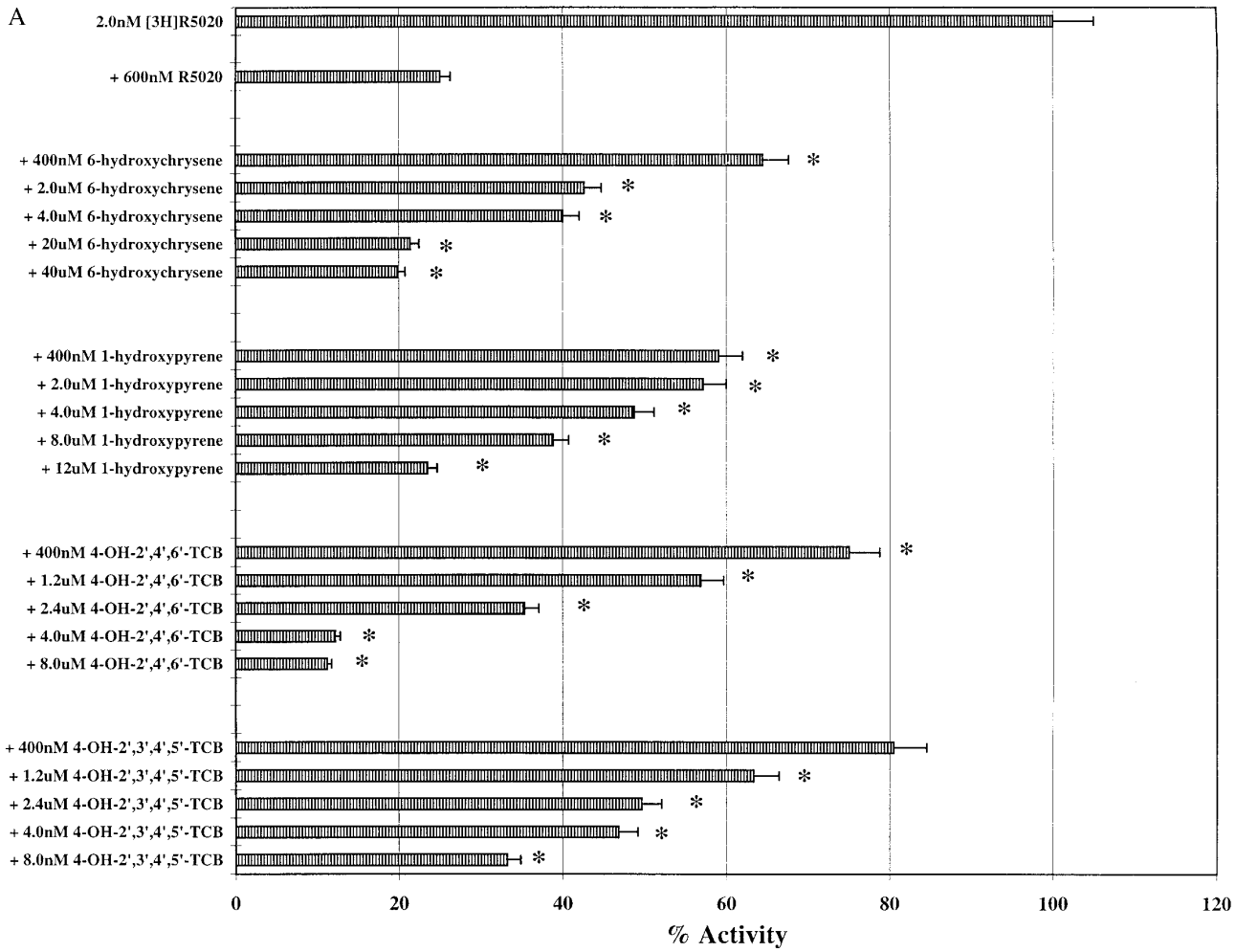

FIG. 3. Competition binding assay in strain hPR-PRE. Strain hPR-PRE was incubated with 2 nM [^{3}H]progesterone in the presence or absence of radioinert progesterone or the indicated chemicals at varying concentrations (A & B). The bound [^{3}H]progesterone was measured by liquid scintillation counting and expressed as percentage of [^{3}H]progesterone in the absence of competitor (1200 dpms). The * indicates % activity that is significantly different from [^{3}H]progesterone.

the inhibitory activities of these compounds closely paralleled their binding hPR.

The inhibitory activities of endosulfan II, endosulfan sulfate and lindane did not correlate with their displacement of [^{3}H]progesterone from hPR. This result could be accounted for by the interaction of these chemicals with a region of hPR that is not responsible for binding [^{3}H]progesterone. A different region of PR is required for binding progesterone compared to the anti-progestin RU486 (18). Nonetheless, endosulfan II, endosulfan sulfate and lindane may inhibit key intermediates in the progesterone signaling cascade in yeast. These chemicals may displace heat shock proteins from hPR and decrease the amount of hPR-progesterone complexes formed. Alternatively, protein kinases which phosphorylate PR may be inhibited after exposure to these chemicals (19).

In conclusion, we have utilized yeast expressing hPR and a progesterone-sensitive reporter to screen a chemical library for hPR agonists and antagonists. This screen indicated that none of the chemicals tested functioned as hPR agonists. Seven chemicals inhibited hPR-mediated transactivation through multiple mechanisms. The list of chemicals which function as hPR antagonists is growing with the recent discovery that nonylphenol, octylphenol, pentachlorophenol (12) and some DDT metabolites (20, 21) can also inhibit progesterone activity *in vitro*. These findings indicate a need for future research aimed at understanding if certain environmental chemicals can alter progesterone-dependent responses *in vivo*.

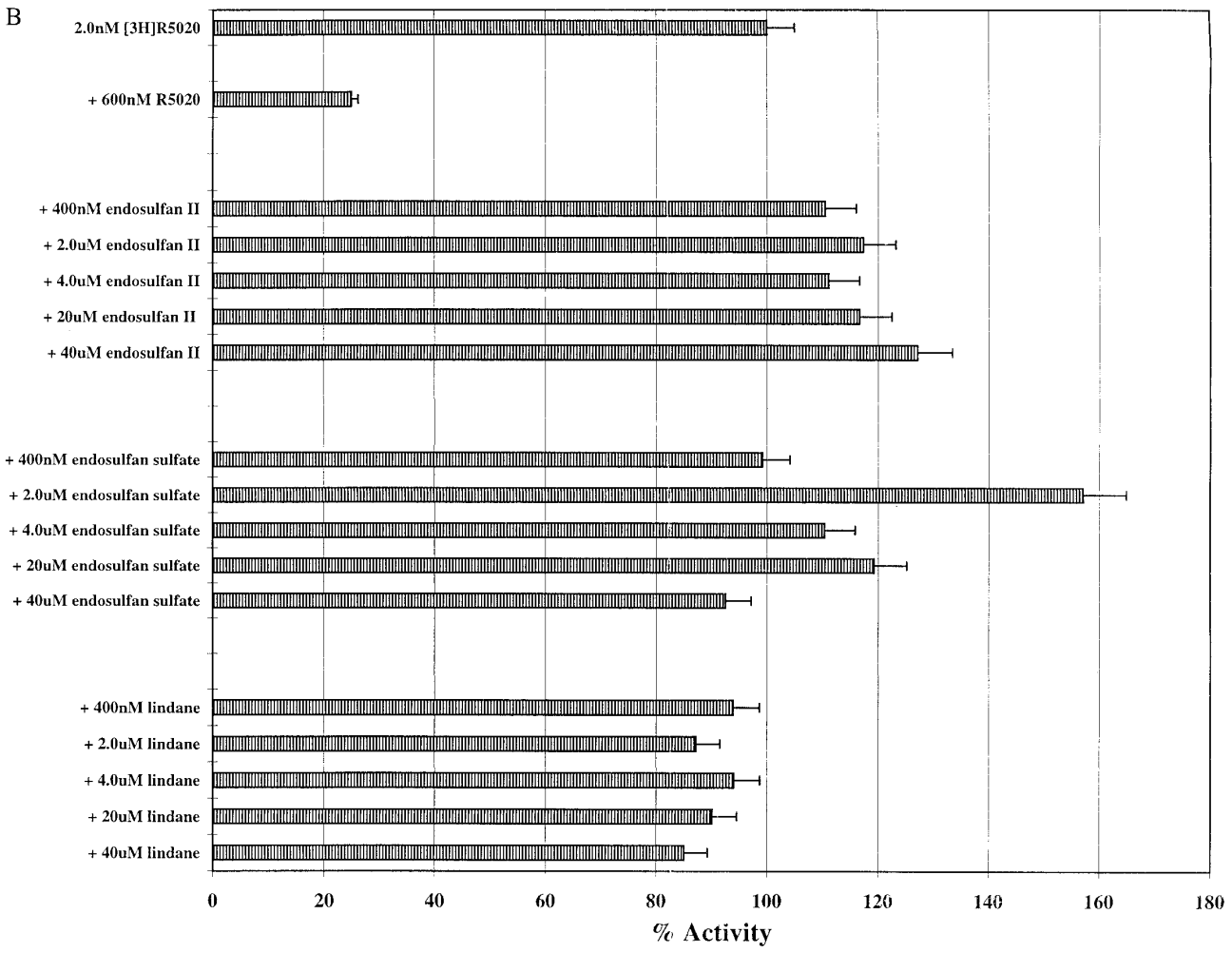

FIG. 3—*Continued*

ACKNOWLEDGMENTS

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