Acute Effects of Polychlorinated Biphenyl-Containing and -Free Transformer Fluids on Rat Testicular Steroidogenesis

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Polychlorinated biphenyl (PCB)-based transformer fluids belong to a class of environmentally persistent mixtures with known toxic effects. Here, we studied the acute effects of Askarel (which contains Aroclor 1260) and two substitute transformer fluids (the silicone oil-based DC 561 and the mineral oil-based ENOL C) on rat testicular steroidogenesis. Single intraperitoneal (ip; 10 mg/kg body weight) or bilateral intratesticular (itt; 25 µg/testis) injections of Askarel markedly decreased serum androgen levels 24 hr after administration. In acute testicular cultures from these animals, choric gonadotropin-stimulated progesterone and androgen productions were severely attenuated. When itt was injected or added in vitro, Askarel inhibited 3β-hydroxysteroid dehydrogenase (3β-HSD), stimulated 17α-hydroxylase/lyase (P450c17), and did not affect 3β-hydroxysteroid dehydrogenase in testicular postmitochondrial fractions. The ip-injected Askarel did not affect 3β-HSD, but inhibited P450c17, suggesting that a more intensive metabolism of peripherally injected Askarel reduces the circulating levels of active ingredients below the threshold needed for inhibition of 3β-HSD and generates a derivative that inhibits P450c17. In contrast to Askarel, itt-injection of DC561 and the mineral oil-based ENOL C did not affect in vivo and in vitro steroidogenesis. These findings show the acute effects of Askarel, but not silicone and mineral oils, on testicular steroidogenesis.

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Polychlorinated biphenyls (PCBs), used in commercial products and found in environmental samples, are complex mixtures of congeners and their degradation products, a feature potentially important in determining the toxicity of a particular mixture. Congeners belong to two major groups: the coplanar PCBs and the ortho-substituted PCBs. The toxic responses of coplanar PCBs are predominantly mediated by activation of the aromatic hydrocarbon (Ah) receptors. Their coupling and actions through Ah receptors resemble those observed with 2,3,7,8-tetrachlorodibenzop-p-dioxin (TCDD), polychlorinated dibenzo-furans (PCDFs), and related substances (1-3). PCDFs, and to a lesser extent TCDD, are found in increasing concentrations in aging PCB mixtures and in samples from heated and/or burned PCBs (2,4-6). The mono-ortho-substituted PCB congeners also exhibit Ah receptor agonist activity (7), but with lower potency than coplanar congeners. The less chlorinated and ortho-substituted PCBs (with two or more ortho chlorines) have low or no affinity for the Ah receptors (8-10). However, they have a profile of hormone and neurotransmitter disrupters that account for their toxicity (9,11).

The biochemical and toxic responses induced by commercial PCBs are diverse and include induction of drug-metabolizing enzymes; thymic atrophy; immuno-, neuro-, dermal, and developmental toxicity; porphyria; and other hepatotoxic effects (7). The interference of PCBs with endocrine and reproductive functions is well documented; most studies have focused on fetal toxicity, developmental malformation, a decrease in reproductive ability, and multiple testicular abnormalities (7,12,13). PCBs exhibit complex estrogenic and antiestrogenic actions that partially account for the observed effects on reproductive functions (14-16). A few studies have also addressed the effects of PCB congeners and mixtures on steroidogenesis in gonadal and adrenal tissues (17-20). In rats, intraperitoneal (ip) injection of a PCB congener, 3,3′,4,4′,5,5′-hexachlorobiphenyl, produces a marked reduction in plasma testosterone concentration (21). A PCB mixture containing only ortho isomers and congeners also inhibits human chorionic gonadotropin (hCG)-induced androgen production in rat interstitial cells (22).

The aim of the present study was to evaluate the acute in vivo and in vitro effects of Askarel, an Aroclor 1260-based transformer fluid, and to compare it with the effects of two substitute fluids, the silicone oil-based DC 561 and the mineral oil-based ENOL C. Our focus was on the effects of these fluids on rat testicular steroidogenesis. We compared the effects of their ip and intratesticular (itt) administration on serum testosterone and in vitro testicular steroidogenesis 24 hr after injection. We also examined the capacity of postmitochondrial testicular fractions from normal animals to produce steroid hormones during a short-term in vitro exposure to Askarel. The results of these investigations indicate that Askarel, but not the substitute fluids, affects testicular steroidogenesis in vivo and in vitro.

Materials and Methods

Chemicals. Askarel (commercial name Pyralene) used in our experiments was supplied by M. Vojinovic-Miloradov, Institute of Chemistry, Novi Sad, Yugoslavia. Chemical characterization of this sample, performed by J. Cochran (Hazardous Materials Laboratory, Champaign, IL, USA), S. Kapor (Laboratory for Biophysics and Analytical Chemistry, Zemun, Yugoslavia), and V. Djordjevic-Milic (Institute for Health Protection, Novi Sad, Yugoslavia), identified Pyralene as Askarel, an Aroclor 1260-based transformer fluid with 10% trichlorobenzene. The silicone-based transformer fluid, Dow Corning 561 Silicone Transformer Liquid (DC 561) was manufactured by Dow Corning Limited (Barry, South Glamorgan, UK), and the mineral oil-based transformer fluid, ENOL C, was manufactured by the Oil Refineries Novi Sad, Yugoslavia. Both samples were obtained from S. Kapor. Antitestosterone-11 boronic acid albumin (BSA; serum no. 250) and antiprogestosterone-11 BSA (serum no. 337) were supplied by G. D. Niswender (Colorado State University, Fort Collins, CO, USA). Medium 199 was purchased from Gibco Laboratories (Gaithersburg, MD, USA). We obtained NADPH, NAD, cytochrome c, BSA (fraction V), collagenease (type 1), testosterone, A4-androstenedione, progesterone, and pregnenolone from Sigma (St. Louis, MO, USA). [1,2,6,73H(N)]-testosterone and [1,2,6,73H(N)]-progesterone were purchased from New England Nuclear (Brussels, Belgium). We purchased Dextran T70 from Pharmacia (Uppsala, Sweden) and charcoal Norit A from Serva (Heidelberg, Germany).

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Tris (hydroxymethyl) aminomethane was purchased from Bethesda Research Laboratories (Bethesda, MD, USA). All other reagents were of analytical grade.

**Animals, treatments, and in vitro steroidogenesis** Male Wistar rats, were raised under controlled environmental conditions (temperature 22 ± 2°C and 14 hr light/10 hr dark) in our laboratory, with food and water ad libitum; animals were used for experiments at approximately 3 months of age. We prepared the desired concentrations of transformer fluids by evaporating the required amount of stock solution and dissolving it in olive oil or saline. Rats were handled daily during a 1-week acclimation period before experiments and then treated with an ip injection of either vehicle (olive oil) or transformer fluid (10 mg/kg body weight [bw]), or with an i.t. injection of either vehicle (saline) or transformer fluid (25 µg/testis), between 0800 and 0830 hr.

We conducted the experiments in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals (23), and the Local Animal Ethical Committee of the Institute of Biology. Animals were sacrificed by decapitation 24 hr after injections, trunk blood was collected, and serum samples were stored at -20°C until analysis for testosterone and dihydrotestosterone (T+DHT) content by radioimmunoassay (RIA). Testes from control and treated rats were quickly removed, decapsulated, weighed, and incubated individually in vials containing 5 mL medium 199 enriched with 0.1% BSA and 20 ng/mL hCG. The contents of the incubation vials were gassed with 95% O2/5% CO2, and incubation was carried out for 3 hr at 34°C in a shaking water-bath (100 oscillations/min). Incubation media was decanted and centrifuged for 10 min at 1,500g, and individual samples of supernatants were stored at -20°C before measurement of androgen (T+DHT) and progesterone levels by RIA.

**Enzyme activities in postmitochondrial fractions** To prepare the postmitochondrial fractions from testicular tissue, testes were decapsulated and homogenized in 50 mM phosphate buffer containing 0.25 M sucrose (pH 7.4), using a glass–glass homogenizer. After centrifugation (4°C for 20 min at 1,500g), the supernatants were mixed with dextran-coated charcoal to remove the endogenous steroids (24). The samples were centrifuged at 1,500g for 10 min, and supernatants were further centrifuged at 12,000g for 20 min. We estimated protein content in postmitochondrial fractions by the Bradford method (25), using BSA as a standard. The desired concentrations of Askarel were prepared by evaporating the necessary amount of stock solution, dissolving it in 0.1 M phosphate buffer, and adding it directly into the corresponding reaction mixture.

We estimated 3β-hydroxysteroid dehydrogenase (3βHSD) activity through the conversion of added pregnenolone to progesterone. The incubation solution, with a final volume of 2 mL, contained 25 µM pregnenolone, 135 µM NADH, 100 mM phosphate buffer (pH 7.4), and 0.1 mL of the postmitochondrial fraction (0.22 mg proteins/tube). Mixtures were incubated for 10 min at 37°C in a shaking water-bath in an atmosphere of 95% O2/5% CO2. We estimated the 17α-hydroxylase/C17,20lyase (P450c17) and 17β-hydroxysteroid dehydrogenase (17βHSD) activities in postmitochondrial fractions by conversion of pregnenolone to testosterone and Δ4-androstenedione to testosterone, respectively (26). Briefly, in the final volume of 0.25 mL, the incubation solution contained 10 µM steroid substrates, 1 mM NADPH, 0.1 M phosphate buffer (pH 7.4), and 0.1 mL postmitochondrial fractions (0.35 mg proteins/tube). Mixtures were incubated for 15 min at 37°C in a shaking water-bath in 95% O2/5% CO2 atmosphere.

We measured 3β[14C] activity in the presence of saturating concentration of pregnenolone (the estimated Km = 8.43 ± 1.76 µM), whereas activities of P450c17 and 17βHSD were measured in the presence of saturated concentrations of corresponding steroid substrates. Selected incubation times were within temporal linearity of the enzyme activities (26,27). Enzyme reactions were initiated by adding 0.1 mL postmitochondrial fractions and terminated by placing the tubes in an ice-cold bath. The samples were stored at -20°C until assayed for testosterone or progesterone by RIA.

We measured NADPH-P450 reductase activity in postmitochondrial fractions of purified interstitial cells as the change in absorbancy at 550 nm (28). Briefly, rat testes were decapsulated and enzymatically dispersed with collagenase according to Anakawe et al. (29), with some modification (27). Interstitial cells were resuspended in 5 mL of 17 mM Tris, 140 mM NH4Cl solution (pH 7.2), and incubated for 10 min at room temperature. This procedure eliminates red blood cells and the interference of hemoglobin. The cell pellets were washed twice with medium 199-BSA and twice with 0.9% saline. Postmitochondrial fractions were prepared as described above. We measured NADPH-P450 reductase activities in 0.1 M phosphate buffer-containing incubation mixtures (final volume 0.6 mL) containing 0.2 mM NADPH, 1 mM KCN, 30 µM cytochrome c, 0.15 mg proteins from postmitochondrial fractions, and increasing concentrations of Askarel or buffer (controls). The reactions were initiated by adding NADPH, which was omitted from the blanks (28,30). The millimolar extinction difference between reduced and oxidized cytochrome c was 21.2 at 550 nm.

**Hormone assays** We estimated androgens and progesterone levels in serum and incubation medium by RIA. Each experiment was run in a single assay. Precision of androgen assay was 6 pg/tube, intra- and interassay coefficients of variation were 5.8% and 7.5%, respectively. The antitestosterone serum used in RIA showed a high cross-reactivity with dihydrotestosterone (DHT), and assay values for androgen production are referred to as T+DHT concentrations. There was no cross-reactivity of progesterone and Δ4-androstenedione with antitestosterone serum in 152± diluted samples used to estimate androgen production in vitro. Precision of progesterone assay was 4 pg/tube and intra-assay and interassay coefficients of variation were 6.8% and 10.7%, respectively. Because antiprogestosterone serum used in RIA assay for progesterone cross-reacts with pregnenolone with a relative binding affinity of 1.4%, additional tubes without postmitochondrial fractions and with pregnenolone were also included, and progesterone production was calculated after subtraction of these blanks for pregnenolone.

To assess the possibility of cross-reactivity between transformer fluids and antiprogestosterone serum used in our RIA assay, we also incubated tubes containing 0.1 M phosphate buffer, corresponding steroid substrate, and increasing concentrations of transformer fluids without postmitochondrial fractions. Transformer fluids did not significantly cross-react with antitestosterone serum (between 0.1% and 1%, depending on added concentration). The cross-reactivity between antiprogestosterone serum and transformers fluids was <1%. The T+DHT and progesterone productions were calculated after subtraction of corresponding blanks for each transformer fluid.

**Statistical analysis.** All results are expressed as means ± SEMs. Statistical analysis for data shown in Figures 2A, 2B, and 4 was performed by Student’s t-test and M ann-Whitney test, with p < 0.05 in both tests. Data shown in Figures 2B and 3B and Tables 1 and 2 were analyzed by analysis of variance (AN OVA), and post hoc comparisons between means were made by D unnett’s test, with p < 0.05.

**Results** Both ip (10 mg/kg bw) and bilateral itt (25 µg/testis) injections of Askarel were associated with a significant decrease in serum T+DHT levels 24 hr after treatments (Figure 1A). The hCG-stimulated androgen production by decapsulated testes from these
animals was also severely inhibited in both experimental groups (Figure 1B), as well as the progesterone production by the same cultures (Figure 1C). These results indicate that Askarel injected in vivo down-regulates serum androgens and the capacity of testicular tissue to synthesize steroid hormones and that this inhibition was independent from the method of exposure (i.e., peripheral versus intratesticular).

To identify the metabolic steps affected by Askarel, in further studies the activities of several steroidogenic enzymes were examined in testicular tissue. The conversion of pregnenolone to progesterone was used to measure the activity of 3β-HSD (see Materials and Methods). As shown in Figure 2A, the activity of this enzyme in testicular postmitochondrial fractions was not changed by ip administration of Askarel (left panel). However, enzyme activity was significantly inhibited in postmitochondrial fractions from itt-treated animals (right panel). Askarel also decreased the conversion of pregnenolone to progesterone when added directly to the postmitochondrial fractions of normal rats (Figure 2B). At the concentrations tested, this inhibition was not dose related, suggesting a high sensitivity of 3β-HSD to Askarel.

The conversion of progesterone to testosterone in postmitochondrial fractions was also affected by Askarel injection (Figure 3). In ip-treated animals, conversion was dramatically inhibited (Figure 3A, left panel). In contrast, this metabolic step was slightly stimulated in postmitochondrial fractions from itt-injected animals (right panel). Consistent with the itt treatment, Askarel added in vitro stimulated the conversion of progesterone to testosterone in a dose-dependent manner (Figure 3B). In general, this stimulatory action of Askarel could be mediated by facilitating the P450c17 pathway, which transforms progesterone to Δ4-androstenedione, or by facilitating 17βH SD, an enzyme that converts Δ4-androstenedione to testosterone. Consistent with the first hypothesis, Askarel added in vitro did not affect the Δ4-androstenedione-supported testosterone production (Table 1).

The observed changes in the P450c17-mediated androgen production could be related to the direct effects of Askarel on this enzyme, or could be indirect, through...
stimulation of NADPH-P450 reductase activity, an enzyme that mediates transport of electrons needed for P450c17 activity. To address the latter possibility, NADPH-P450 reductase activity was measured in postmitochondrial fractions of interstitial cell preparations from adult rats 15 min after incubation with Askarel. As shown in Table 2, Askarel increased the activity of NADPH-P450 reductase when added at 11 ppm. However, in concentrations that affected the conversion of progesterone to testosterone, Askarel was ineffective (Figure 3B), indicating that P450c17 was directly stimulated.

Samples of DC561 and ENOL C transformer fluids were tested by the itt route of administration, and results of these investigations are presented in Figure 4. Both transformer fluids had no effect on serum androgen levels (Figure 4A) and hCG-stimulated androgen production by decapsulated testes (Figure 4B). Also, no changes in the conversion of pregnenolone to progesterone (Figure 4C), progesterone to testosterone (Figure 4D), and Δ4-androstenedione to testosterone (not shown) by postmitochondrial fractions were observed. This suggests that the activities of 317H SD, 17βH SD, and P450c17 were unchanged in DC561 and ENOL C-treated animals.

Discussion

The potential toxic effects of PCB-containing and PCB-free transformer fluids on steroidogenesis have not been studied previously. However, several reports have addressed the effects of variable PCB mixtures on steroidogenic enzymes. For example, Aroclor 1248 down-regulates rat testicular steroidogenesis by an acute inhibition of 317H SD and P450c17 (31). This mixture contains 21% of trichlorobiphenyl, 55% of tetrachlorobiphenyl, and 21% of pentachlorobiphenyl, each of which have similar amounts of mono-ortho and di-ortho congeners (32). In contrast, Aroclor 1254 does not alter the activity of P450c17 in the microsomal fraction of guinea pig testes when injected in vivo or added in vitro (20). This commercial mixture of PCB congeners contains about 2% of trichlorobiphenyl, 17% of tetrachlorobiphenyl, 49% of pentachlorobiphenyl, 29% of hexachlorobiphenyl, and 4% of octachlorobiphenyl (32).

Here we studied the acute in vivo and in vitro effects of Askarel, an Aroclor 1260-based transformer fluid, and the two substitute PCB-free transformer fluids, DC561 and ENOL C. Aroclor 1260 contains 47% of hexachlorobiphenyl and 37% of heptachlorobiphenyl, and between them more than 85% of congeners with two or more ortho-chlorines (32). Our results indicate that Askarel inhibited rat testicular steroidogenesis when administrated in vivo. Independently of the mode of exposure (ip vs. itt), injection of Askarel was accompanied by a strong reduction of serum androgen levels. In parallel to changes observed in blood, a significant inhibition of hCG-stimulated progesterone and androgen production by decapsulated testes was observed.

We also measured the activities of several steroidogenic enzymes in postmitochondrial fractions of in vivo-treated animals by following the conversion of corresponding substrate to a certain product (see Materials and Methods). The effects of Askarel on steroid conversion were complex. Similar to the effects of Aroclor 1248 (31), Askarel also inhibited 317H SD activity in the postmitochondrial fractions from both itt-treated animals and when added in vitro. In contrast to Aroclor 1248 (31) and Aroclor 1254 (20), Askarel facilitated P450c17 activity in the same experimental conditions. This facilitation probably occurs through direct action because NADPH-P450 reductase activity was affected only in high Askarel concentrations. Finally, 17βH SD, an enzyme that converts Δ4-androstenedione to testosterone, was not affected. Thus, it is likely that the mixture of PCBs may or may not exhibit a specific toxic effect, depending on the presence of a particular compound and mixture of certain congeners.

Because the participation of aromatase, a P450-dependent enzyme, on steroidogenesis in whole cell testis homogenates and the potential effects of Askarel on this enzyme were not tested, we cannot exclude the impact of this pathway on the estimated T+DHT levels in our experiments. However, the ratio of testosterone versus estradiol levels in adult rat testes is more than 1,000 (33), suggesting that aromatization as a possible pathway in metabolism of testicular testosterone is of minor significance in the 3-month-old animals used in our experiments. Also, TCDD induced no effect on aromatase activity in rat ovary, although it affected the activity of P450c17 (34). Therefore, it is highly probable that the impact of aromatase activity and the potential modulation of its activity by Askarel do not significantly affect T+DHT levels measured in the whole cell testis homogenates during 3-hr incubation period.

The effects of Askarel on steroid conversion also depended on the type of administration. No inhibition of 317H SD activity was observed in ip-treated animals, whereas Askarel inhibited this enzyme when injected itt and added in vitro. In contrast, the conversion of progesterone to testosterone was inhibited in ip-treated animals, whereas itt-treated animals and in vitro-added Askarel stimulated conversion. Such differences could

Figure 4. Effects of itt-injection of silicon oil-based DC561 and mineral oil-based ENOLC transformer fluids on (A) T+DHT levels, (B) in vitro hCG (20 ng/ml)-stimulated androgen production, (C) the conversion of pregnenolone to progesterone, and (D) progesterone to androgens in postmitochondrial fractions. Groups of rats were treated by a bilateral itt-injection (25 µg/testis) of silicon or mineral oil and animals were sacrificed 24 hr after injection. Bars represent mean ± SEM. In (A) the numbers above the bars represent the number of animals; in (B), (C), and (D), the numbers above the bars represent the number of testes or individual testicular postmitochondrial fractions from control and treated rats in one of two similar experiments. *p < 0.05 versus corresponding controls.
be explained by a more intensive metabolism of Askarel in the liver when injected ip, compared to that after itt application. Thus, the former difference may result from a reduction in the circulating levels of active ingredients present in Askarel below the threshold needed for inhibition of 3ph D. It is also known that metabolism of PCBs results in the formation of various derivatives, many of which are toxic (16), which may provide a rationale for down-regulation of P450c17 activity in ip-injected animals.

Concentrations of Askarel used in our in vitro studies were in the range of 2.2 ppb to 2.2 ppm, whereas general levels of PCBs in soil and sediment are in the range of parts per billion, and in water in subparts per trillion (33). PCBs are found consistently in many environmental matrices, including marine plants and animals, freshwater fish, freshwater soils, air and water. For example, the estimated contents of PCBs in samples of carp and pike taken from the Tisza, Sava, and Danube rivers in Europe were in the range of 9–25 ppb and 11–37 ppb, respectively (36). Because the allowed concentrations of PCBs in food samples are in the 0.3–5 ppm range (35), the sensitivity of testicular androgenesis to PCB mixture in the subnanomolar to nanomolar concentration ranges is of potential importance for environmental contamination and altered reproductive function.

On the other hand, the two commonly used substitute transformer fluids, DC561 and ENOL C, did not affect rat testicular steroidalogenesis. M ineral oils are complex mixtures of oligaphic hydrocarbons, naphthenic, and aromatics, the relative distribution of which depends on the source of the oil and method of refinement (37). In general, the belief that such composition should not be toxic is consistent with our results. However, used and recycled mineral oils exhibit mutagenic and carcinogenic effects (37,38). It would be of interest to compare the effects of used and nonused samples of silicone- and mineral-oil-based transformer fluids on rat testicular steroidalogenesis. Also, it is possible that such fluids have different target mechanisms compared to PCBs.

In summary, this study indicates that the Aroclor 1260 based-transformer fluid Askarel inhibits rat testicular steroidalogenesis in vivo and in vitro applications. 3BH D activity is inhibited by Askarel when added in vitro or itt-injected, but is not affected when transformer fluid is injected ip. The effect of Askarel on P450c17 activity depended on the type of application; there is inhibition after ip application, and even a stimulatory effect after itt application and when added in vitro. NADPH-P450 reductase activity was also slightly affected by Askarel, as well as a step(s) before proges-
trone formation. In all experimental condi-
tions, Askarel and/or degradable metabolites of this mixture influenced testicular steroidaloge-


type. These findings provide a rationale for a number of observations on the antagonal actions of PCBs and suggest that both DC561 and ENOL C express no endocrine-
disrupting activity.

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