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Differential effects of octylphenol, 17β-estradiol, endosulfan, or bisphenol A on the steroidogenic competence of cultured adult rat Leydig cells

Eisuke P. Murono*, Raymond C. Derk, Jesús H. de León

Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Pathology and Physiology Research Branch, M/S L-2015, 1095 Willowdale Road, Morgantown, WV, 26505-2888, USA

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Abstract

In the current studies, we evaluated the effects of 4-tert-octylphenol (OP), endosulfan, bisphenol A (BPA), and 17β-estradiol on basal or hCG-stimulated testosterone formation by cultured Leydig cells from young adult male rats. Exposure of Leydig cells to increasing concentrations of OP (1 to 2000 nM), 17β-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM) or BPA (1 to 1000 nM), alone or with 10 mIU/mL hCG for 4 or 24 h, did not lower ambient testosterone levels, although cells exposed to higher OP concentrations + hCG for 24 h often had modest declines in testosterone (10 to 20%). Of interest, exposure to the highest concentration OP (2000 nM) alone for 4 or 24 h increased testosterone levels (~ 2-fold in 4-h exposed cells). Whether prior exposure to OP + hCG for 24 h affects the subsequent conversion of steroid substrates to testosterone over 4 h was evaluated. Progressive declines in 1 μM 22(R) hydroxycholesterol, 1 μM pregnenolone, or 1 μM progesterone conversion to testosterone was observed beginning at 100 to 500 nM OP exposure (maximal declines of 40 to 12% of controls were observed); however, the conversion of 1 μM androstenedione to testosterone was not affected by OP. These results suggested that 24-h exposure to OP + hCG has no effect on 17β-hydroxysteroid dehydrogenase, which converts androstenedione to testosterone, but that it inhibits the 17α-hydroxylase/C17–20 lyase step, which converts progesterone to androstenedione. In addition, potentially, OP could inhibit cholesterol side/chain cleavage activity, which converts cholesterol to pregnenolone, and/or 3β-hydroxysteroid dehydrogenase, which converts pregnenolone to progesterone. Of interest, exposure to increasing concentrations of 17β-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM) + hCG for 24 h had no effect on subsequent conversion of 22(R)hydroxycholesterol to testosterone. Furthermore, the inhibiting effects of OP + hCG exposure on subsequent conversion of progesterone to testosterone was unaffected by concomitant exposure to the pure estrogen antagonist, ICI 182,780, or the antioxidants, ascorbate or dimethyl sulfoxide, suggesting that the actions of OP are not mediated through binding to estrogen receptor α or β or by free radical induced damage to steroidogenic enzymes, respectively. These results demonstrate that direct exposure of adult Leydig cells to OP may have subtle effects on their ability to produce testosterone, which may not be detected by measuring ambient androgen levels. In addition, the effects of OP on Leydig cell testosterone formation appear to be different from those of the native estrogen, 17β-estradiol, and from other reported weak xenoestrogens such as endosulfan and BPA.

Keywords: Cultured adult Leydig cells; Xenoestrogens; Testosterone biosynthesis

1. Introduction

Recent publications have hypothesized that environmental/occupational exposure to endocrine-disrupting chemicals have had adverse effects on the reproductive system of humans and various wildlife species [1,2]. Changes that have occurred in males over the past 40 to 50 years attributed to gestational exposure to these chemicals [3] include 1) an international decline in semen quality [4], 2) a rise in the incidence of cryptorchidism [5], and 3) an increase in the incidence of testicular cancer [6]. However, this idea is not universally accepted [7], and it has not been established whether such changes have had any impact on male fertility [8]. Although there is disagreement on the extent to which exposure to endocrine-disrupting agents has adversely altered male reproductive functions, there is general agree-

* Corresponding author. Tel.: +1-304-285-6145; fax: +1-304-285-5938.

E-mail address: eem8@cdc.gov (E.P. Murono).

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ment on the need for additional studies to clarify the association between exposure and potential effects on human reproductive health [9].

Alkylphenol ethoxylates (APEs) are a class of nonionic surfactants widely used in the manufacture of detergents, paints, pesticides, and plastics [10]. The alkyl groups primarily are branched nonyl or octyl chains positioned opposite the para-substituted ethoxylate chain, composed of one to 100 repeating ethylene oxide units. 4-Tert-octylphenol (octylphenol, OP) and 4-nonylphenol (NP) are environmentally persistent degradation products of APEs [11]. Both OP and NP have been reported to mimic the effects of estrogen in various testing assays. Thus, they were demonstrated to stimulate growth [12] and to displace ^{3}H-estradiol binding to the estrogen receptor [13] of MCF-7 human breast cancer cells, to stimulate estrogen-dependent β-galactosidase activity in a yeast estrogen screen assay [14], and to stimulate uterine growth when administered in vivo to immature rats [15]. Bisphenol A (BPA) is a monomer used in the manufacture of polycarbonate and epoxy resins. Similar to OP and NP, BPA has been reported to exhibit estrogen-like activity in both in vitro [16] and in vivo [17] test systems. Endosulfan is a polychlorinated cyclodiene insecticide widely used in agriculture [18], and it has been reported also to have estrogenic effects when applied to estrogen-sensitive MCF-7 cells [19].

It has been reported that inappropriate gestational exposure to estrogenic chemicals can adversely affect reproductive function of both male and female offspring. Male offspring of mothers treated with diethylstilbestrol (DES), a synthetic estrogen used in the 1950s and 1960s to prevent pregnancy complications, were reported to have a higher incidence of testicular hypoplasia, cryptorchidism, and semen abnormalities [20]. Similarly, male offspring of pregnant mice exposed to DES were shown to have a higher incidence of sterility [21] and cryptorchidism [22], and gestational exposure of pregnant rats to DES or OP was reported to decrease the amount of the steroidogenic enzyme 17α-hydroxylase/C17–20 lyase (P450c17) and of steroidogenic factor 1 (SF-1), a transcription factor involved in the development of adrenals and gonads and the expression of steroidogenic enzymes [23,24]. In addition, exposure of neonatal rats to NP reduced the size of the testis and accessory sexual organ weights and increased the incidence of cryptorchidism [25]. In adult rats, chronic exposure to OP or estradiol valerate was reported to decrease testis size and sperm numbers [26]. With respect to testicular effects of BPA, maternal exposure of pregnant mice (days 11 through 17 of gestation) to BPA reduced efficiency of sperm production (number of spermatids per gram testis) in young adult males [27]. With respect to the gonadal effects of endosulfan, oral exposure of young adult male rats to endosulfan was reported to decrease circulating testosterone levels [28], and in immature males, it was reported to reduce testicular spermatid numbers [29].

The testicular effects of OP and BPA are proposed generally to mimic the actions of estrogen; however, the mode of action of endosulfan on the testis has not been determined. In addition, whether the actions of each of these chemicals on the testis represent direct or indirect effects, and their specific mechanism and site(s) of action have not been established. Using an in vitro adult rat Leydig cell model, we examined the potential direct effects of OP, BPA, or endosulfan on basal or hCG-stimulated testosterone formation, and we compared the effects of these chemicals to that of 17β-estradiol.

2. Materials and methods

2.1. Animals

Young adult male Sprague-Dawley (Hla: (SD) CVF) rats were purchased from Hilltop Lab Animals, Inc., Scottdale, PA, USA. Animals were housed in polycarbonate shoebox cages (two per cage) and exposed to a 12 h light and 12 h dark cycle. They were fed Purina rat chow (R-M-H 3500 with 5% fat content) and provided tap water ad libitum. Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All animal protocols were reviewed and approved by the local animal studies committee.

2.2. Reagents

Collagenase (Type I), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNase I), 22(R)-hydroxycholesterol, 5-pregnen-3β-ol-20-one (pregnenolone), 4-pregnen-3,20-dione (progesterone), 4-androsten-3,17-dione (androstenedione), L-ascorbic acid, dimethyl sulfoxide (Me_2SO), 8- bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin (BSA, clinical reagent grade) and Ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA. Dulbecco's Modified Eagle Medium without phenol red (DMEM), F-12 Nutrient Mixture without phenol red (F-12), Medium 199 (Med 199), Hank's balanced salt solution without Ca^{2+} and Mg^{2+}(HBSS), sodium bicarbonate, soybean trypsin inhibitor, and N-2-hydroxyethylpiperazine-NR-2- ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY, USA. [2,3,6,7-^{3}H(N)]-Testosterone (specific activity $\sim$ 100 Ci/mmol) and [^{125}I]-human chorionic gonadotropin (^{125}I- hCG, specific activity $\sim$ 50 μCi/mg) were from NEN Life Science Products, Boston, MA, USA. 17β-Estradiol and testosterone were from Steraloids, Wilton, NH, USA. ICI 182,780 (ICI, pure antiestrogen) was a gift from Dr. A.E. Wakeling (Zeneca Pharmaceuticals, Cheshire, England). 4-tert-Octylphenol (OP) and bisphenol A (BPA) were from Aldrich Chemical Company, Milwaukee, WI, USA. Endosulfan was from Chem Service, West Chester,

PA, USA. Percoll was from Pharmacia, Piscataway, NJ, USA. Human chorionic gonadotropin (hCG, CR-127, specific activity 14900 IU/mg) was a gift from NIDDK, Bethesda, MD, USA.

2.3. Isolation and culture of Leydig cells

Animals were 55 to 65 days of age when sacrificed by intraperitoneal (i.p.) injection of pentobarbitol. To obtain sufficient cells for culture, testes from 8 to 10 animals were utilized. Testes were decapsulated and digested in 0.25 mg/mL collagenase in Med 199 + 0.1% BSA and 10 μg/mL DNase I for 20 to 30 min at 37°C. The dispersed interstitial cells were elutriated using a Beckman elutriation apparatus as described previously [30]. Cells retained in the elutriation chamber were layered over a 60% Percoll gradient and centrifuged at $\sim$ 25,000 *g* for 1 h. Leydig cells localized between densities of 1.07 and 1.09 g/mL. These cells were $\sim$ 95% pure based on histochemical staining for 3β- hydroxysteroid dehydrogenase (3β-HSD) activity.

Leydig cells were resuspended in a 1:1 mixture of DMEM/F-12 without phenol red containing 15 mM HEPES (pH 7.4), 15 mM $NaHCO_3$, 100 U/mL penicillin G, 100 μg/mL streptomycin and 0.1% BSA as described previously [31]. One milliliter of cells (1×10^5/mL) was added into each 1.6-cm diameter well of a 24-well Costar culture plate and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 33°C; however, for ^{125}I-hCG binding studies, 1 ml of cells containing 2×10^5/mL was added. Fresh medium without BSA was added $\sim$ 20 h after plating, and treatments were initiated. Cells were exposed to various treatments for 4 or 24 h.

2.4. Treatment of cells

OP and 17β-estradiol were dissolved in ethanol. The final ethanol concentration in all treatment groups (including controls) was 0.1%. BPA and endosulfan were dissolved in Me_2SO The final Me_2SO concentrations for each chemical (including controls) were 0.1 and 0.15%, respectively. These concentrations of ethanol or Me_2SO did not affect testosterone biosynthesis or cell viability. Furthermore, the concentrations of OP, 17β-estradiol, BPA, and endosulfan used in these experiments did not affect cell viability based on cell morphology, attachment to culture plates, or the exclusion of trypan blue.

2.5. Quantitation of testosterone formation, ^{125}I-hCG binding to LH receptors and cellular cAMP levels

Testosterone was quantitated directly from the culture medium by radioimmunoassay (RIA) as described previously [32]. Quantitation of ^{125}I-hCG binding to LH receptors was similar to a procedure described for cultured immature rat Leydig cells [33]. Cellular cAMP content of cultured Leydig cells was quantitated after acetylation by RIA (Amersham cAMP kit, no. RPA 509) as described previously [34].

2.6. Statistical analysis

Data were expressed as mean $\pm$ SEM and analyzed by ANOVA. Differences among treatment means were determined using Student-Newman-Keuls' test. A P value $<$ 0.05 was considered statistically significant.

3. Results

3.1. Effect of increasing concentrations of OP, alone or in the presence of 10 mIU/ml hCG, on testosterone formation following 4 or 24 h exposures

The testosterone level following 4 h of culture in control cells was 6.9 $\pm$ 0.4 ng/10^5 cells (Fig. 1, panel A). Exposure to 1 to 500 nM OP did not alter testosterone levels; however, exposure to 2000 nM OP significantly increased testosterone to 13.1 $\pm$ 1.2 ng/10^5 cells. Control Leydig cells produced 8.1 $\pm$ 0.7 ng testosterone/10^5 cells following 24 h of culture (Fig. 1, panel B). Exposure to 1 to 100 nM OP did not alter testosterone levels; however, exposure to 500 or 2000 nM OP increased testosterone to 11.2 $\pm$ 0.5 and 12.7 $\pm$ 0.4 ng/10^5 cells, respectively. In response to exposure to hCG alone for 4 h, Leydig cells produced 91.0 $\pm$ 3.0 ng testosterone/10^5 cells (Fig. 1, panel C). Exposure to OP (1 to 2000 nM) did not have a significant effect on hCG-stimulated testosterone. In response to exposure to hCG alone for 24 h, Leydig cells produced 127.9 $\pm$ 7.0 ng testosterone/10^5 cells (Fig. 1, panel D). Exposure to OP (1 to 2000 nM) did not have a significant effect on hCG-stimulated testosterone. In 11 separate experiments testosterone levels were 100 to 76% of control in cells exposed to 2000 nM OP + hCG, and in 2 of these experiments, testosterone levels were significantly less than control.

3.2. Effect of increasing concentrations of 17β-estradiol, endosulfan or BPA, alone or in the presence of 10 mIU/ml hCG, on testosterone formation following 4 or 24 h exposures

Exposure to increasing concentrations of 17β-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM) for 4 h or 24 h and without or with hCG, had no effect on testosterone levels (data not shown).

3.3. Effects of increasing concentrations of OP, endosulfan or BPA on 1 mM 8 Br-cAMP-stimulated testosterone following 24 h exposure

Increasing concentrations of OP (1 to 2000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM) did not

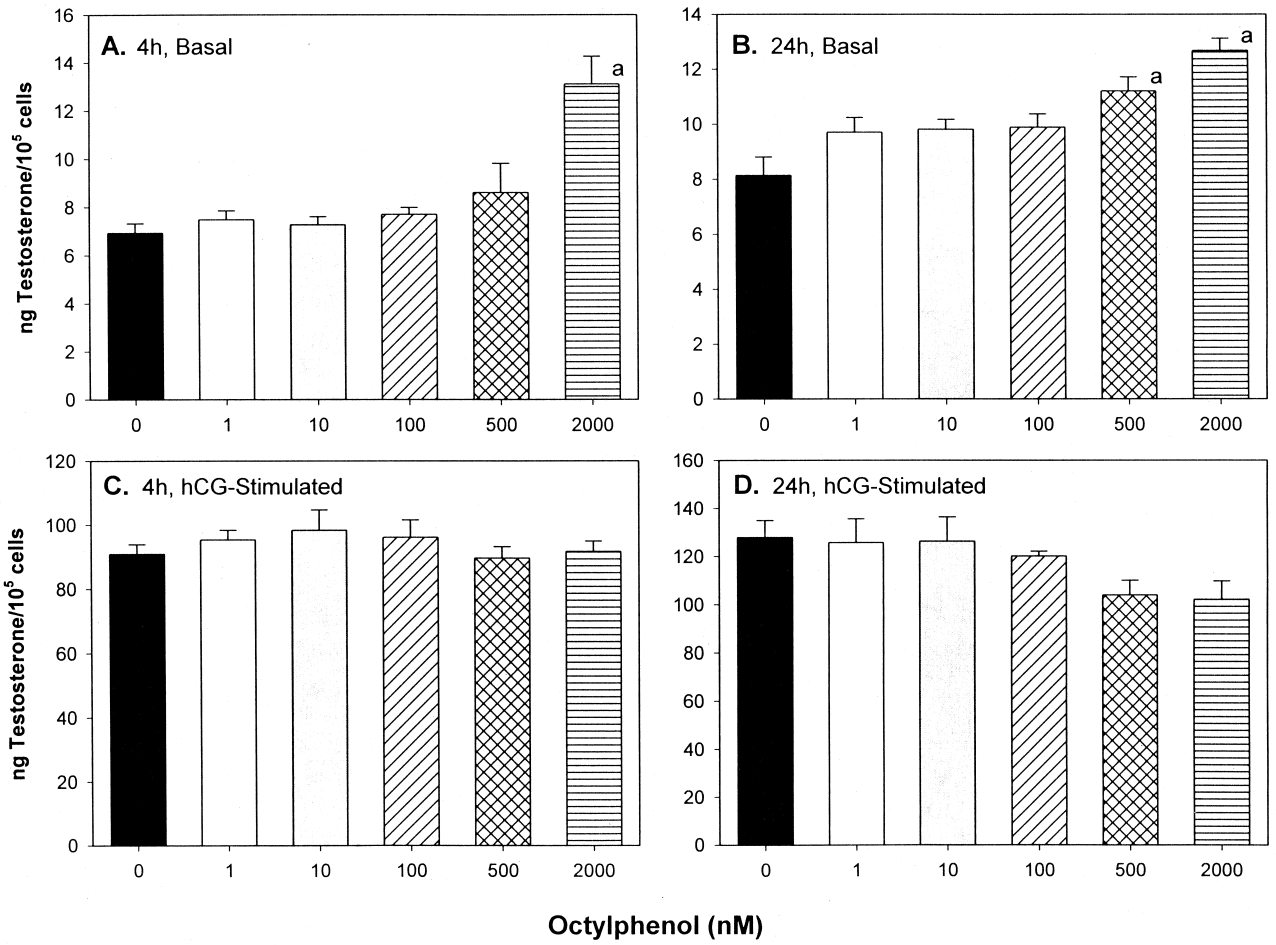

Fig. 1. Effect of octylphenol on testosterone formation of cultured adult Leydig cells. Leydig cells were exposed to increasing concentrations of OP (1 to 2000 nM) alone or with 10 mIU/mL hCG for 4 or 24 h. Each treatment represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiements. [a] $P < 0.05$ when compared to appropriate control.

significantly alter 1 mM 8 Br-cAMP-stimulated testosterone levels following 24 h of exposure (data not shown).

3.4. Conversion of steroid precursors to testosterone over 4 h following initial 24 h exposure to increasing concentrations of OP and 10 mIU/ml hCG

Although testosterone levels were not significantly reduced in cultured Leydig cells following exposure to increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG, the possibility that specific steroidogenic enzymes converting cholesterol to testosterone might be affected following this exposure was evaluated. Following the initial 24-h exposure to increasing OP concentrations and hCG, fresh media containing 1 μM 22(R)hydroxycholesterol was added to each well, and its conversion to testosterone after incubation for 4 h was evaluated. A progressive decline in the conversion 22(R)hydroxcholesterol conversion testosterone was observed at the 2 highest concentrations of OP (Fig. 2, panel A). The conversion of cholesterol to testosterone involves 4 enzymatic steps: 1) cytochrome P-450 cholesterol side-cleavage (P450scc), which converts cholesterol to pregnenolone; 2) 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD), which converts pregnenolone to progesterone; 3) 17α-hydroxylase/c17–20-lyase (P450c17), which converts progesterone to 17α-hydroxyprogesterone and androstenedione; and 4) 17β-hydroxysteroid dehydrogenase (17β-HSD), which converts androstenedione to testosterone.

The conversion of 1 μM pregnenolone or progesterone to testosterone over 4 h following initial exposure to increasing concentrations of OP and hCG exhibited a pattern similar to that of 22(R)hydroxycholesterol (Fig. 2, panels B and C, respectively), although with pregnenolone as substrate, the decline in testosterone with 100 nM OP was significant. In contrast, the conversion of 1 μM androstenedione to testosterone over 4 h after the initial 24 h exposure to increasing OP concentrations and hCG was unaffected (Fig. 2, panel D). Collectively, this pattern of steroid substrate conversion to testosterone suggests that 17β-HSD activity is unaffected by OP, while P450c17 activity appears to be inhibited by OP. In addition, P450scc

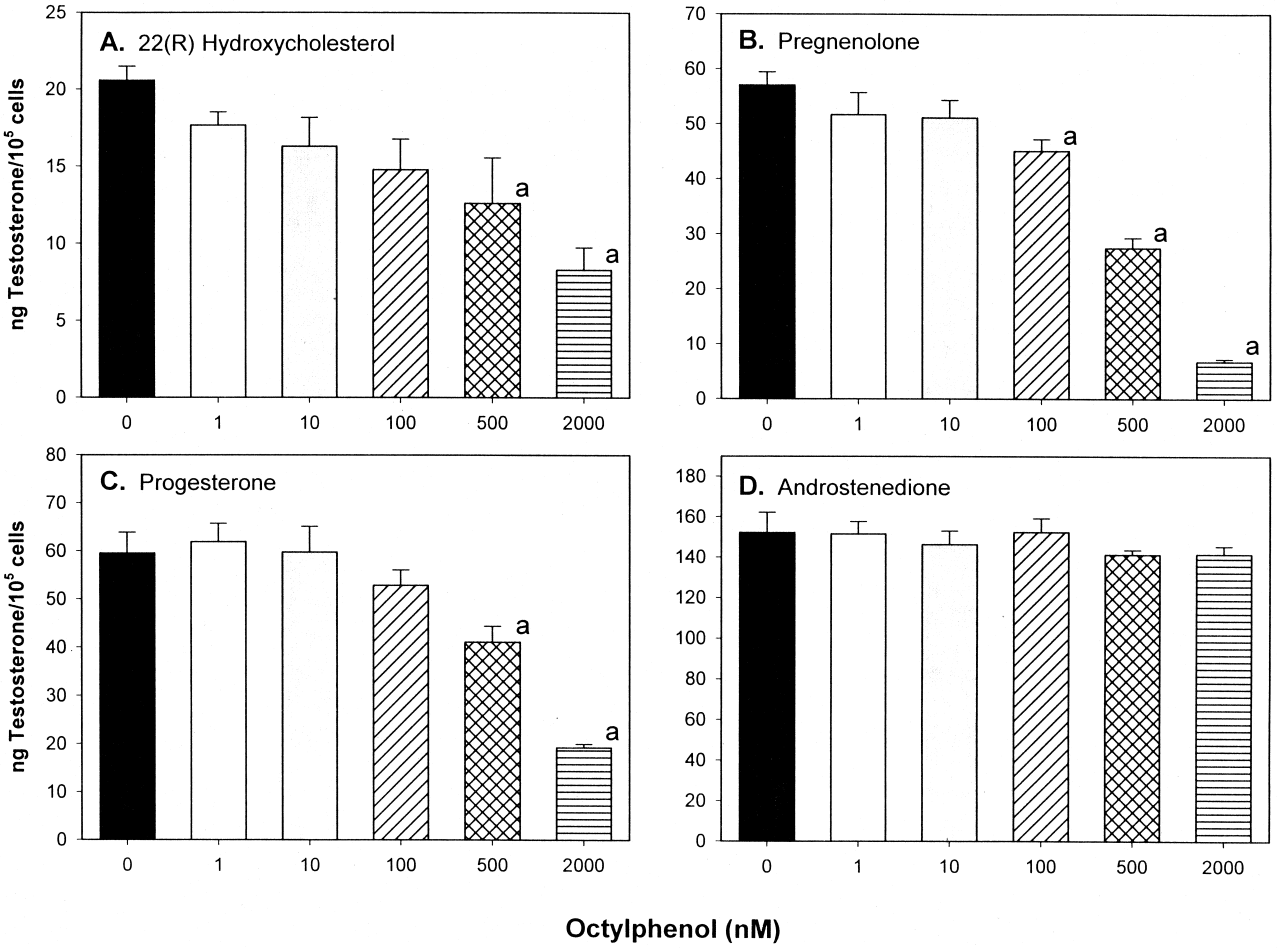

Fig. 2. Effect of initial exposure to octylphenol and hCG on subsequent conversion of steroid substrates to testosterone. Leydig cells were exposed to increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h. Next, fresh medium containing 1 μM 22(R)hydroxycholesterol (panel A), 1 μM pregnenolone (panel B), 1 μM progesterone (panel C), or 1 μM androstenedione (panel D) was added to each well, and testosterone formation after incubation for 4 h was determined. Each treatment represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiments. $^{a}P < 0.05$ when compared to appropriate control.

and/or 3β-HSD activities may be inhibited by OP, but the pattern of testosterone biosynthesis using 22(R) hydroxycholesterol or pregnenolone as substrates could be ascribed to the inhibition of P450c17. The conversion of 22(R) hydroxycholesterol or progesterone to testosterone over 4 h following an initial exposure to increasing concentrations of OP alone or with hCG for 4 h or of increasing concentrations of OP alone for 24 h, also was evaluated. Testosterone levels were no different than control under each of these conditions, suggesting that the increase in testosterone following exposure to OP alone required the continued presence of OP or that the enhancement of another segment of the steroidogenic pathway is responsible for the increase in androgen, and that in the presence of hCG, more than 4 h of exposure is needed to exert the inhibiting effects of OP on 22(R) hydroxycholesterol or progesterone conversion to testosterone.

3.5. Conversion of 1 μM 22(R)hydroxycholesterol to testosterone over 4 h following initial 24 h exposure to increasing concentrations of 17β-estradiol, endosulfan, or BPA, and 10 mIU/ml hCG

Exposure to increasing concentrations of 17β-estradiol (1 to 1000 nM) + hCG for 24 h had no effect on the subsequent conversion of 22(R)hydroxycholesterol to testosterone (Fig. 3, panel A). Similarly, exposure to increasing concentrations of endosulfan (1 to 1000 nM) or BPA (1 to 1000 nM) + hCG for 24 h had no effect on the subsequent conversion of 22(R)hydroxycholesterol to testosterone (Fig. 3, panels B and C, respectively). Thus, in contrast to OP, exposure to 17β-estradiol, endosulfan, or BPA does not appear to alter the activity of any of the steroidogenic enzymes involved in the conversion of cholesterol to testosterone in cultured adult Leydig cells.

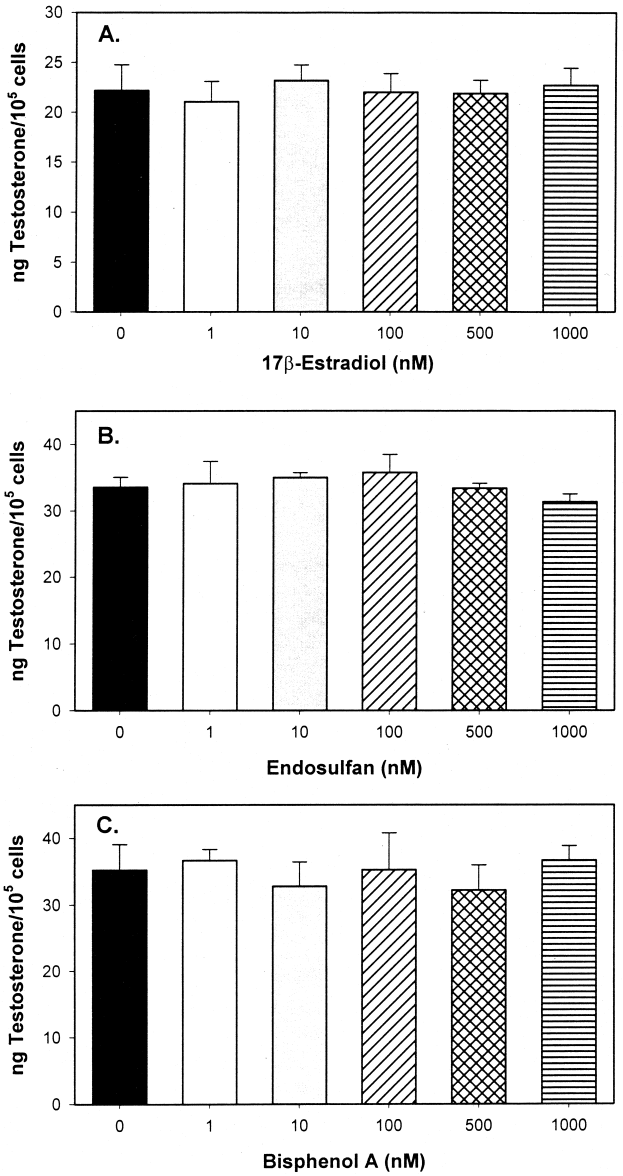

Fig. 3. Effect of initial exposure to 17β-estradiol, endosulfan, or bisphenol A and hCG on subsequent conversion of 22(R)hydroxycholesterol to testosterone. Leydig cells were exposed to increasing concentrations of 17β-estradiol (1 to 1000 nM, panel A), endosulfan (1 to 1000 nM, panel B), or BPA (1 to 1000 nM, panel C), and 10 mIU/mL hCG for 24 h. Next, fresh medium containing 1 μM 22(R)hydroxycholesterol was added to each well, and testosterone formation after incubation for 4 h was determined. Each treatment represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiments.

3.6. Effect of concomitant exposure to 1 μm ICI 182,780 + increasing concentrations of OP + 10 mIU/mL hCG for 24 h on subsequent conversion of 1 μM progesterone to testosterone over 4 h

To evaluate whether the actions of OP in inhibiting the conversion of progesterone to testosterone is mediated through binding to estrogen receptor α or β, 1 μM ICI 182,780, a pure estrogen antagonist, was included during the initial 24 h exposure to increasing OP concentrations and hCG. Concomitant incubation of cultured Leydig cells with ICI 182,780 did not alter the decline in the conversion of progesterone to testosterone by exposure to higher OP concentrations (Fig. 4).

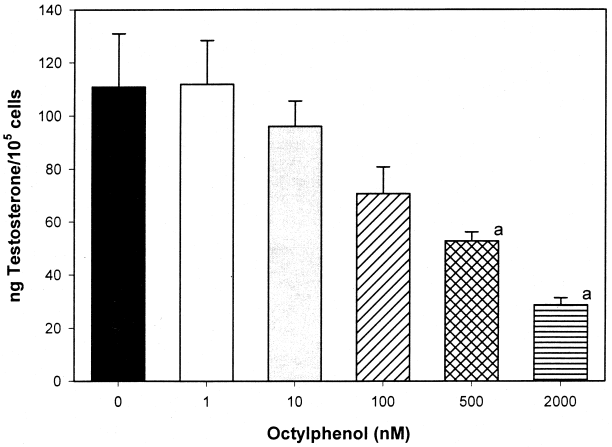

Fig. 4. Effect of initial concomitant exposure to ICI 182,780, octylphenol, and hCG on subsequent conversion of progesterone to testosterone. Leydig cells were exposed to 1 μM ICI 182,780, increasing concentrations of OP (1 to 2000 nM) and 10 mIU/ml hCG for 24 h. Next, fresh medium containing 1 μM progesterone was added to each well, and testosterone formation after incubation for 4 h was determined. Each treatment represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiments. $^{a}P <$ 0.05 when compared to control.

3.7. Effect of concomitant exposure to 0.2 mM ascorbate or 10 mM Me_2SO and increasing concentrations of OP + 10 mIU/ml hCG for 24 h on subsequent conversion of 1 μM progesterone to testosterone over 4 h

The possibility that OP could function as a pseudosubstrate and thereby lead to the generation of oxygen free radicals which can cause lipid peroxidation and damage to steroidogenic enzymes was examined. The inclusion of 0.2 mM ascorbate or 10 mM Me_2S0 to cells exposed for 24 h to increasing OP concentrations + hCG had no effect on the inhibition of progesterone conversion to testosterone (Fig. 5, panels A and B, respectively). The inclusion of both ascorbate and Me_2SO to OP + hCG exposed cells, similarly, had no protective effects on progesterone conversion to testosterone (data not shown).

3.8. Effect of increasing concentrations of OP, endosulfan, or BPA on ^{125}I-hCG binding to Leydig cells

Because exposure of Leydig cells to hCG down regulates luteinizing hormone (LH) receptors [35], exposed cells were treated with OP (1 to 2000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM) alone for 24 h prior to quantitating ^{125}I-hCG binding to LH receptors. Exposure to increasing concentrations of OP, endosulfan, or BPA alone

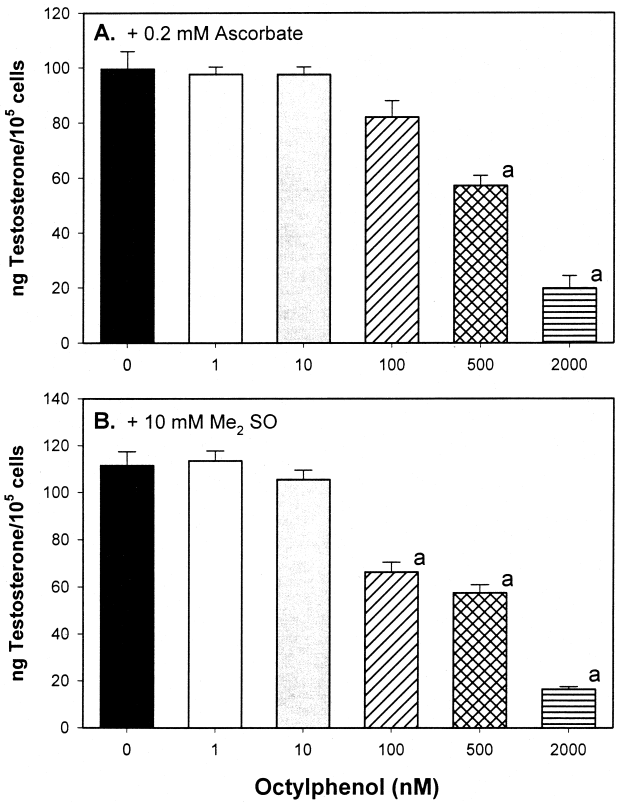

Fig. 5. Effect of initial concomitant exposure to antioxidants, octylphenol and hCG on subsequent conversion of progesterone to testosterone. Leydig cells were exposed to 0.2 mM ascorbate (panel A) or 10 mM Me_2SO (panel B) and increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h. Next, fresh medium containing 1 μM progesterone was added to each well, and testosterone formation after incubation for 4 h was determined. Each treatment represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiments. $^{a}P < 0.05$ when compared to appropriate control.

for 24 h had no effect on ^{125}I-hCG binding to LH receptors in Leydig cells (data not shown).

Whether exposure to increasing concentrations of OP, endosulfan or BPA, alone or with 10 mIU/mL hCG, for 4 or 24 h, affect cellular cAMP levels also was evaluated. Cellular cAMP levels were unaffected by any of these treatments (data not shown).

4. Discussion

The studies described herein demonstrate that the sensitivity of cultured adult rat Leydig cells to OP with respect to testosterone biosynthetic competence differs from that to 17β-estradiol, the native secreted estrogen, and the xenoestrogens endosulfan and BPA. Although treatment with increasing concentrations of OP (1 to 2000 nM), 17β-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM), alone or with 10 mIU/mL hCG for 4 or 24 h did not significantly lower testosterone levels released into the medium, exposure to 100 to 500 nM and higher of OP + hCG for 24 h inhibited the subsequent conversion of 22(R) hydroxycholesterol, pregnenolone, or progesterone (but not androstenedione) to testosterone over 4 h. In contrast, initial exposure of Leydig cells to increasing concentrations of 17β-estradiol, endosulfan or BPA + hCG for 24 h had no effect on the subsequent conversion of 22(R)hydroxycholesterol to testosterone over 4 h. Furthermore, concomitant exposure of Leydig cells to the pure estrogen antagonist ICI 182,780, increasing concentrations of OP and 10 mIU/mL hCG for 24 h did not alter the subsequent inhibition of progesterone conversion to testosterone, suggesting that the effects of OP are not mediated through the estrogen receptor. These results suggest that in vitro OP has the potential to inhibit one or more enzymatic steps involved in the conversion of cholesterol to androstenedione and that the effects of OP on hCG-stimulated testosterone biosynthesis differ from that of the native estrogen and from other reported weak estrogenic chemicals (endosulfan and BPA).

We reported previously that exposure of cultured neonatal rat Leydig cells (which represent a “fetal” population of Leydig cells) to increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h had a biphasic effect on testosterone formation, with lower concentrations (1 and 10 nM) actually increasing testosterone levels (10 to 70% above control), while higher OP concentrations (100 to 2000 nM) progressively reduced testosterone from peak levels [31]. Exposure to increasing concentrations of OP alone for 4 or 24 h or with 10 mIU/mL hCG for 4 h, in contrast, had no or very little effect on testosterone formation. In both cultured precursor Leydig cells and Leydig cells from immature rats (23 days of age), exposure to increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h caused a progressive decline in testosterone formation beginning at 100 or 500 nM OP and higher [34]. Thus, there are differences in the pattern or sensitivity of testosterone response to direct OP exposure of Leydig cells from animals of different ages. Currently, we cannot explain why these differences in sensitivity to direct OP exposure exist. However, this may be explained, in part, by the fact that neonatal (fetal) Leydig cells represent a completely separate population of steroidogenic cells that actively produce testosterone during the later stages of gestation [36] but then become nonfunctional after about 2 to 3 weeks following birth in rodents [37]. Precursor Leydig cells are derived from mesenchymal cells localized within the interstitium of immature testes, and they differentiate into immature Leydig cells between the second and fourth weeks after birth [38]. Immature Leydig cells differentiate into adult Leydig cells after about 40 days following birth [39]. Thus, because each of these cell types represent distinct stages of Leydig cell maturation, their responses and/or sensitivity to OP may differ.

The pattern of steroid substrate conversion to testosterone over 4 h after an initial 24 h exposure to OP (1 to 2000

nM) + hCG (no effect on androstenedione conversion to testosterone, but inhibition of 22(R)hydroxycholesterol, pregnenolone or progesterone conversion to testosterone), suggests that 17β-HSD activity is unaffected by OP and that P450c17 activity is inhibited by OP. Potentially, P450scc and/or 3β-HSD activities also could be inhibited by OP in adult Leydig cells, but inhibition of P450c17 activity alone could account for the pattern of testosterone formation observed following addition of 22(R)hydroxycholesterol or pregnenolone as substrates. In future studies, it will be necessary to measure P450scc and 3β-HSD activities specifically to determine whether OP also affects these enzymes. In a study reported previously, exposure of cultured precursor or immature rat Leydig cells to OP (1 to 2000 nM) + hCG for 24 h inhibited P450c17 activity but not 17β-HSD activity as observed in the present study [34]. However, in that study ambient testosterone levels of cells exposed to OP + hCG for 24 h were reduced, suggesting that precursor and Leydig cells from immature rats may be more sensitive to OP than adult Leydig cells. In contrast to the pattern of steroid substrate conversion to testosterone observed in cultured precursor and immature Leydig cells from prepubertal rats and adult Leydig cells following initial exposure for 24 h to increasing OP concentrations and hCG, the conversion of 22(R)hydroxycholesterol, pregnenolone, or progesterone conversion to testosterone was unaffected in cultured Leydig cells from neonatal rats [31], suggesting that the site(s) of sensitivity of neonatal Leydig cells to OP differed from that of older Leydig cells.

In one other in vitro study using cultured mouse Leydig Tumor cells (mLTC-1), exposure to BPA or OP was reported to inhibit hCG-stimulated progesterone production [40]. These cells lack the enzymatic capacity to convert progesterone to testosterone, so that progesterone is the main steroid product secreted. In contrast to the current studies where the primary actions of OP occurred between the enzymatic steps converting cholesterol to androstenedione, in mLTC-1 cells, the main actions of OP occurred prior to the P450scc step (due to a decline in cAMP formation) and, furthermore, these cells were sensitive to BPA [40]. These differences in sensitivity to BPA and OP and the site(s) of sensitivity to OP may be due to species differences (mouse vs rat cells) and cell type (tumor cell vs primary cell culture).

There is some similarity of the current results with recent studies on the effects of polychlorinated biphenyls (PCBs), a mixture of persistent industrial chemicals used in the manufacture of hydraulic and transformer fluids, on rat Leydig cell androgen formation. PCBs have been reported to exhibit both estrogenic and antiestrogenic activities in various assays [41], and, in addition, some of the actions of PCBs appear to be mediated through the arylhydrocarbon receptor (AhR). In interstitial cells from adult rats, a mixture of PCBs was reported to inhibit hCG-stimulated or progesterone-supported (but not androstenedione-supported) testosterone formation following exposure for 2 h, suggesting that P450c17 activity was inhibited by PCBs [42]. A subsequent study demonstrated that another mixture of PCBs (Aroclor 1248) inhibited testicular 3βHSD activity following 24-h in vivo exposure or following 2-h exposure of intact interstitial cells [43]. Aroclor 1248 also inhibited 3βHSD and P450c17 activities following 10 to 15 min exposure of a postmitochondrial fraction of testes from normal adult rats [43]. However, the complexity of the Leydig cell response to PCBs was demonstrated by the observation that Askarel (containing Aroclor 1260, another mixture of PCBs), stimulated P450c17 activity when added directly to a post mitochondrial fraction of testes from normal adult rats [44]. It is not known whether OP and PCBs alter testosterone by Leydig cells by similar or different mechanisms; however, it will be important to determine the specific mode of action of each chemical to better understand the potential cummulative effect of exposure to a mixture of these and similar chemicals on steroidogenic competence.

With respect to how the inhibiting effects of OP on cholesterol conversion to testosterone are mediated, the possibility that OP could inhibit testosterone production by acting as a pro-oxidant was examined. Two cytochrome P450 enzymes (P450scc and P450c17) are involved in converting cholesterol to testosterone in Leydig cells. They use molecular oxygen and electrons from NADPH for substrate hydroxylation. During normal steroidogenesis, reactive oxygen species (superoxide and/or hydroxyl radicals) are produced by electron leakage outside the electron transfer chains [45,46], and these oxygen radicals can initiate lipid peroxidation to inactivate P450 enzymes [47]. Antioxidants such as ascorbate and Me_2SO are protective against superoxide and hydroxyl radicals, respectively [45,48]. The inability of ascorbate or Me_2SO to protect against the inhibitive effects of OP in the current studies suggest that OP does not inhibit adult Leydig cell steroidogenesis by generating free radicals. Similar to the current studies, antioxidants were unable to protect against the inhibitive effect of OP on testosterone formation in cultured precursor and Leydig cells from immature rats [34].

Although we were able to identify steroidogenic deficits in the capacity of adult rat Leydig cells to convert cholesterol to androstenedione (by focusing on a specific locus of the steroidogenic pathway), it should be recognized that hCG-stimulated testosterone levels in the ambient media of exposed cells were not reduced significantly following the initial exposure for 24 h with OP + hCG, suggesting that in adult animals, exposure to OP may not dramatically lower circulating testosterone levels. That this may be the case is suggested by recent multigeneration in vivo studies where continuous exposure through the diet to NP [49] or OP [50] produced limited effects on the reproductive system of male rats, although in both studies potential changes in circulating hormone levels (gonadotropins or androgens) were not measured. Although dietary exposure of rats to NP or OP (up to 2000 ppm) appears to have limited adverse effects on

the reproductive system, both i.p. or s.c. exposures have been reported to have adverse reproductive effects on males [24–26]. These observations are supported by a recent study that examined the uterotropic response of immature rats to OP administered by gavage vs s.c. injection [51]. This study found that the uterotropic response was greater following s.c. injection of OP than following exposure via the oral route.

An interesting observation in the current studies was the nearly 2-fold increase in testosterone levels following exposure to increasing concentrations of OP alone. This pattern was not observed in neonatal Leydig cells [31] or immature Leydig cells [34]. Because adult Leydig cells in situ would be expected to be exposed continuously to circulating LH, the physiologic relevance of this observation is not clear. Nevertheless, this observation illustrates the potential varied actions of OP on Leydig cells, which distinguishes it from the effects of 17β-estradiol or other reported xenoestrogens.

References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect. 1993;101:378–84.

[2] Neubert D. Vulnerability of the endocrine system to xenobiotic influence. Regula Toxicol Pharmacol 1997;26:9–29.

[3] Sharpe RM, Skakkebaek NE. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341:1392–5.

[4] Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. British Med J 1992;305:609–13.

[5] John Ratcliffe Hospital Cryptorchidism Study Group. Cryptorchidism. A prospective study of 7500 consecutive male births. 1985–8. Arch Dis Childhood 1992;67:892–903.

[6] Brown LM, Pottern LM, Hoover RN, Devesa SS, Aselton P, Flannery JT. Testicular cancer in the United States: trends in incidence and mortality. Intl J Epidermiol 1986;15:164–70.

[7] Safe SH. Environmental and dietary estrogens and health: Is there a problem? Environ Health Perspect 1995;103:346–51.

[8] Lamb DJ. Hormonal disruptors and male infertility: Are men at serious risk? Regula Toxicol Pharmacol 1997;26:30–3.

[9] Cooper RL, Kavlock RJ. Endocrine disruptors and reproductive development: a weigh-of- evidence overview. J Endocrinol 1997;152: 159–66.

[10] Nimrod AC, Benson WH. Environmental estrogenic effects of alkylphenol ethoxylates. Crit Rev Toxicol 1996;26:335–64.

[11] Naylor CG, Mieure S, Hoare SA, Sumpter JP, Parker MG. Alkylphenol ethoxylates in the environment. JAOCS 1992;69:695–705.

[12] White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 1994;135:175–82.

[13] Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts relative *in vitro* bioactivity of xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 1997;105: 70–6.

[14] Arnold SF, Robinson MK, Notides AC, Guillette LT, Jr., McLachlan JAA yeast estrogen screen for examining the relative exposure of cells to natural, and xenoestrogens. Environ Health Perspect 1996; 104:544–8.

[15] Odum J, Lefevre PA, Tittensor S, Paton D, Routledge EJ, Beresford NA, Sumpter JP, Ashby J. The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regula Toxicol Pharmacol 1997;25:176–88.

[16] Krushnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Encocrinology 1993;132:2279–86.

[17] Ashby J, Tinwell H. Uterotrophic activity of bisphenol A in the immature rat. Environ Health Perspect 1998;106:719–20.

[18] Naqvi SM, Valshnavi C. Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals. Comp Biochem Physiol 1993;1050:347–61.

[19] Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 1994;102:380–3.

[20] Gill WB, Schumacher GFB, Bibbo M, Straus FH, Schoenberg HW. Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. J Urol 1979;122: 36–9.

[21] McLachlan J, Newbold R, Bullock B. Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 1975;190: 991–2.

[22] Nomura T, Kanzaki T. Induction of urogenital anomalies and some tumors in the progeny of mice receiving diethylstilbestrol. Cancer Res 1977;37:1099–1104.

[23] Majdic G, Sharpe RM, O'Shaughnessy PJ, Saunders PTK. Expression of cytochrome P450 17α-hydroxylase/C17–20-1yase in the fetal rat testis is reduced by maternal exposure to exogenous estrogen. Endocrinology 1996;137:1063–70.

[24] Majdic G, Sharpe RM, Saunder PTK. Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor -1 (SF-1/ Ad4BP) in fetal rat testis. Mol Cell Endocrinol 1997;127:91–8.

[25] Lee PC. Disruption of male reproductive tract development by administration of the xenoestrogen, nonylphenol to male newborn rats. Endocrine 1998;9:105–11.

[26] Boockfor FR, Blake CA. Chronic administration of 4-tert-octylphenol to adult male rats cause shrinkage of the testes and male accessory organs, disrupts spermatogenesis, and increases the incidence of deformities. Biol Reprod 1997;57:267–77.

[27] vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV. A physiologically based approach to the study of bisphenol A, and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Indust Health 1998;14:239–60.

[28] Singh SK, Pandey RS. Effect of sub-chronic endosulfan exposures on plasma gonadotrophins, testosterone and enzymes of androgen biosynthesis in rat. Indian J Exp Biol 1990;28:953–6.

[29] Sinha N, Narayan R, Saxena DK. Effect of endosulfan on the testis of growing rats. Bull Environ Contam Toxicol 1997;58:79–86.

[30] Klinefelter GR, Hall PF, Ewing LL. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. Biol Reprod 1987;36:769–83.

[31] Murono EP, Derk RC, de Leon JH. Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats. Reprod Toxicol 1999;13:451–62.

[32] Murono EP, Lin T, Osterman J, Nankin HR. The effects of cytochalasin B on testosterone synthesis by interstitial cells of rat testis. Biochim Biophys Acta 1980;633:228–36.

[33] Murono EP, Washburn AL, Goforth DP, Wu N. Biphasic effect of basic fibroblast growth factor on ^{125}I-human chorionic gonadotropin binding to cultured immature Leydig cells. Mol Cell Endocrinol 1993;92:121–6.

[34] Murono EP, Derk RC, de Leon JH. Octylphenol inhibits testosterone biosynthesis by cultured precursor and immature Leydig cells from rat testes. Reprod Toxicol 2000;14:275–88.

[35] Leon MD, Calvo JC, Charreau EH, Chemes HE. Developmental changes in testicular interstitial cell populations, LH receptors and in the response to hCG in the rat. Intl J Androl 1990;13:110–22.
[36] Picon R, Gangneau M-N. Acquisition of sensitivity to LH in relation to fetal development. Stimulation of cyclic AMP and testosterone production in the rat testis. Mol Cell Endocrinol 1980;18:137–50.
[37] Kuopio T, Tapanainen J, Pelliniemi, LJ, Huhtaniemi I. Developmental stages of fetal-type Leydig cells in prepubertal rats. Development 1989;107:213–20.
[38] Hardy MP, Zirkin BR, Ewing LL. Kinetic studies on the development of the adult population of Leydig cells in testes of pubertal rat. Endocrinology 1989;124:762–70.
[39] Corpechot C, Baulieu E-E, Robel P. Testosterone, dihydrotestosterone, and androstanediols in plasma, testes, and prostates of rats during development. Acta Endocrinol 1981;96:127–35.
[40] Nikula H, Talonpoika T, Kaleva M, Toppari J. Inhibition of hCG-stimulated steroidogenesis in cultured mouse Leydig tumor cells by bisphenol A and octylphenols. Toxicol Applied Pharmacol 1999;157: 166–73.
[41] Fielden MR, Chen I, Chittim B, Safe SH, Zacharewski TR. Examination of the estrogenicity of 2,4,6,2′,6′-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2′,6′- pentachloro-4-biphenyl (HO-PCB 104) and a further chlorinated derivative 2,4,6,2′,4′,6′-hexachlorobiphenyl (PCB 155). Environ Health Perspect 1997;105: 1238–48.
[42] Kovacevic R, Vojinovic-Miloradov M, Teodorovic I, Ahdric S. Effect of PCBs on androgen production by suspension of adult rat Leydig cells *in vitro.* J Steroid Biochem Molec Biol 1995;52:595–7.
[43] Andric SA, Kostic TS, Stojilkovic SS, Kovacevic RZ. Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture Aroclor 1248. Biol Reprod 2000;62:1882–8.
[44] Andric SA, Kostic TS, Dragisic SM, Andric NL, Stojilkovic SS, Kovacevic RZ. Acute effects of polychlorinated biphenyl-containing and -free transformer fluids on rat testicular steroidogenesis. Environ Health Perspect 2000;108:955–9.
[45] Hornsby PJ, Crivello JF. The role of lipid peroxidation and biological oxidations in the function of the adrenal cortex. Mol Cell Endocrinol 1983;30:1–20.
[46] Hanukoglu I, Rapoport R, Weiner L, Sklan D. Electron leakage from the mitochondrial NADPH-adrenodoxin reductase-adrenodoxin-P450scc (cholesterol side chain cleavage) system. Arch Biochem Biophys 1993;305:489–98.
[47] Hornsby PJ. Regulation of cytochrome P-450-supported 11β-hydroxylation of deoxycortisol by steroids, oxygen and antioxidants in adrenocortical cell cultures. J Biol Chem 1980;255:4020–7.
[48] Hornsby PJ. Cytochrome P-450/pseudosubstrate interactions, and the role of antioxidants in the adrenal cortex. Endocrine Res 1986;12: 469–94.
[49] Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Mintz N, Wolfe G. The effects of 4-nonylphenol in rats: a multigeneration reproduction study. Toxicol Sci 1999;52:80–91.
[50] Tyl RW, Meyers CB, Marr MC, Brine DR, Fail PA, Seely JC, van Miller JP. Two-generation reproduction study with para-tert-octylphenol in rats. Regula Toxicol Pharmacol 1999;30:80–95.
[51] Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 2000;54:154–67.