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Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats

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Abstract

The present studies evaluated the suitability of using cultured dispersed testicular cells from neonatal rats as a source for fetal Leydig cells and the use of these cells to examine direct toxic effects of environmental/occupational chemicals on androgen biosynthesis. For the current studies, the direct actions of octylphenol (OP), a surfactant additive widely used in the manufacture of various detergents, on testosterone biosynthesis by cultured rat neonatal Leydig cells were examined. Octylphenol is considered a xenoestrogen and has been reported to mimic the actions of estrogen in many cellular systems. Following exposure of cultured cells for 24 h to varying concentrations of OP (1 to 2000 nM) together with 10 mIU/mL human chorionic gonadotropin (hCG), the lower concentrations of OP (1 and 10 nM) consistently enhanced testosterone levels (approximately 10 to 70% above control), whereas higher OP concentrations (100 to 2000 nM) progressively decreased testosterone from peak levels to approximately 40 to 80% below control at the highest OP concentration. Interestingly, increasing concentrations of 17β-estradiol (1 to 1000 nM) were without effect on testosterone biosynthesis under the same conditions, and the biphasic pattern of testosterone biosynthesis elicited by increasing OP concentrations was unaffected by concomitant treatment with 10 or 100 nM ICI 182,780, which is considered a pure estrogen antagonist. Therefore, the actions of OP on testosterone biosynthesis by cultured neonatal Leydig cells do not appear to be mediated through the classic estrogen receptor α or β pathway. Although the increase in testosterone levels after exposure to lower OP concentrations and to 0.1 and 1.0 mM 8-Br-cAMP was attenuated, suggesting that lower OP concentrations may alter cellular cAMP levels, because hCG-stimulated cAMP levels were unaffected by any of the OP concentrations evaluated, it appears that its main site(s) of action occurs after the generation of cAMP. In addition, because pretreatment of cells with increasing OP concentrations and hCG had no effect on the conversion of steroid precursors (22(R)-hydroxycholesterol, pregnenolone, progesterone, or androstenedione) to testosterone, it seems that the main actions of OP under the present conditions occur before the mitochondrial cholesterol side-chain cleavage step. Furthermore, because concomitant treatment of cells with various antioxidants (α-tocopherol, butylated hydroxyanisole, or ascorbic acid) did not alter the biphasic pattern of testosterone response to increasing concentrations of OP and hCG, it seems that OP is not acting as an anti- or pro-oxidant in producing these effects. It will be important to determine whether this dose-sensitive response to OP is observed in vivo, and whether the maturational status of Leydig cells influences their pattern of response to OP and similar chemicals. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Octylphenol; Testosterone; Neonatal Leydig cell

1. Introduction

There has been considerable recent interest over the proposal that environmental/occupational exposure to endocrine-disrupting chemicals may alter the normal functioning of the endocrine system of humans and various wildlife species and thereby have adverse reproductive consequences [1]. Several reproductive changes have been reported over the past 40 to 50 years in males, including: 1) a world-wide decline in semen quality [2], 2) an increase in the prevalence of cryptorchidism [3] and 3) an increase in the incidence of testicular germ cell cancer [4]. Although these changes have been linked to an increased exposure in utero to estrogen-mimicking chemicals [5], this association has not been universally accepted [6]. Furthermore, whether these changes have an impact on male fertility has not been established [7]. Nevertheless, there is general agreement on a need for additional in vivo and in vitro studies to under-

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stand more fully the nature of the effects of endocrine-disrupting chemicals and their mechanisms of action in altering male reproductive functions [8].

Alkylphenol ethoxylates (APEs) are a class of nonionic surfactants widely used in the manufacture of agricultural, industrial, and household detergents, as well as in the manufacture of paints, herbicides, pesticides, and plastics [9]. The primary alkyl groups are branched nonyl or octyl chains located opposite the para-substituted ethoxylate chain, composed of one to 100 repeating ethylene oxide units. 4-Tert-octylphenol (octylphenol, OP) and 4-nonylphenol are degradation products of APEs, and they have been reported to be environmentally persistent [10]. Although not originally manufactured to mimic estrogens, OP was reported to stimulate growth of estrogen-responsive MCF-7 human breast cancer cells [11], to displace ^{3}H-estradiol binding to the estrogen receptor of MCF-7 cells [12], and to stimulate estrogen-dependent β-galactosidase activity in a yeast estrogen screen (YES) assay [13].

Previous studies have suggested that inappropriate exposure of males to chemicals with estrogenic activity during fetal development can adversely affect reproductive functions. Thus, male offspring of mothers treated with diethylstilbestrol (DES), a synthetic estrogen used in the 1950s and 1960s to prevent complications of pregnancy, were reported to have a higher incidence of cryptorchidism, testicular hypoplasia, and semen abnormalities [14]. In addition, male offspring of pregnant mice treated with DES have been reported to have a higher incidence of sterility [15] and cryptorchidism [16]. More recently, treatment of pregnant rats with DES or OP was reported to decrease the amount of the steroidogenic enzyme P450 17α-hydroxylase/C17–20-lyase (P450c17) in the fetal testes detected by immunocytochemistry or measured biochemically [17]. Similarly, maternal exposure to DES or OP was reported to reduce the expression of steroidogenic factor 1 (SF-1/Ad4BP), a transcription factor involved in the development of adrenals and gonads and in the expression of steroidogenic enzymes [18], in fetal rat Sertoli and interstitial cells [19]. In adult rats, chronic treatment with OP or estradiol valerate was reported to reduce testis size and sperm numbers [20]. Because these effects of OP or estrogen were evaluated in intact animals, it was unclear whether their actions were due to direct or indirect effects on the testis.

Rat fetal Leydig cells appear and begin to secrete testosterone on approximately Day 15 of gestation [21,22]. Steroid content per Leydig cell is highest during fetal life, suggesting that fetal Leydig cells have greater steroidogenic competence than adult type Leydig cells [23]. Although testosterone secreted by fetal Leydig cells is essential for the differentiation of the internal reproductive structures (both ductal system and accessory sexual glands) and external genitalia in humans, in rodents it is synthesized relatively later during gestation and appears mainly to stabilize these structures. Fetal Leydig cells persist for 2 to 3 weeks after birth and remain steroidogenically competent [23,24]; however, thereafter they regress and are replaced by an adult-type Leydig cell [25,26]. Accordingly, in the present studies fetal Leydig cells were isolated from testes of neonatal rats (6 to 7 days of age), and cultured to evaluate their suitability as a model to examine direct toxic effects of OP on their steroidogenic competence and to compare the effects of OP with those of 17β-estradiol.

2. Materials and Methods

2.1. Animals

One- to 3-day-old neonatal Sprague-Dawley rats with nursing mothers were purchased from Hilltop Lab Animals, Inc., Scottdale, PA. Animals were housed in shoebox cages and exposed to a 12 h light and 12 h dark cycle. Mothers were fed standard rat chow and provided with 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 approved and reviewed by the local animal studies committee.

2.2. Reagents

Collagenase (Type I), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNAse I), etiocholan-3β-ol-17-one, 4-pregnen-3,20-dione (progesterone), 5-pregnen-3β-ol-20-one (pregnenolone), 4-androsten-3,17-dione (androstenedione), 22(R)-hydroxycholesterol, L-ascorbic acid, butylated hydroxyanisole, α-tocopherol, 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. Bovine serum albumin (BSA, clinical reagent grade) and Ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals, Inc., Costa Mesa, CA. Dulbecco's Modified Eagle Medium (DMEM, without phenol red), F-12 Nutrient Mixture (F-12, without phenol red), Medium 199, Hank's balanced salt solution (HBSS, without Ca^{++} and Mg^{++}), sodium bicarbonate, soybean trypsin inhibitor, and N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY. [1, 2, 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. The cAMP RIA kit was from Amersham, Arlington Heights, IL. Tissue culture plates (24-well, 1.6 cm diameter) were from Corning/Costar, Cambridge, MA. 17β-Estradiol and testosterone were from Steraloids, Wilton, NH. ICI 182,780 (pure antiestrogen) was a gift from Dr A.E. Wakeling (Zeneca Pharmaceuticals, Cheshire, England). Octylphenol (4-tert) was from Aldrich Chemical Co., Milwaukee, WI. Percoll was from Pharmacia, Piscataway, NJ. Human chorionic gonadotropin (hCG, CR-127,

specific activity 14,900 IU/mg) was a gift from NIDDK, Bethesda, MD.

2.3. Isolation of Leydig cells from neonatal rats

Neonatal rats (6 to 7 d of age) were sacrificed by exposure to CO_2. Testes were removed, decapsulated, and digested for ~45 min at 37°C with 0.25 mg/mL collagenase in Medium 199 containing 0.1% BSA (Med 199-BSA) and 10 μg/mL DNAse I. The digestion was stopped by addition of cold Med 199-BSA, and the dispersed cells were separated from the seminiferous tubules by gravity sedimentation of the heavier intact tubules. The dispersed cells were washed with Med 199-BSA and layered over a 60% Percoll gradient. The gradient was centrifuged for 1 h at 12,000 *g* at 4°C, and cells localizing between densities of 1.052 and 1.068 g/mL were isolated. This step removes the heavier red blood cells and the lighter germ cells, and the isolated cells represent ~20% neonatal (fetal) Leydig cells based on positive staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) [27]. Leydig cells were washed in Med 199-BSA, then 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 for plating as described previously [28].

2.4. Culture of Leydig cells from neonatal rats

Leydig cells (10^5 in 1 mL of media) were plated into 1.6 cm diameter, 24-well Costar culture plates and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 33°C. Two days after plating, fresh medium (lacking BSA) was added, and treatments were initiated. Cultures were maintained for 4 h to 7 d depending on the experiment, with media change and retreatment every other day of culture for studies extending beyond 2 d, unless stated otherwise.

2.5. Treatment of cells

17β-Estradiol and OP were dissolved in ethanol. The final concentration of ethanol in all treatment groups (including controls) was 0.1%. This concentration of ethanol did not affect testosterone biosynthesis by cultured neonatal Leydig cells or cell viability. Furthermore, none of the concentrations of chemicals tested had any effect on cell viability based on cell morphology and attachment to culture plates.

2.6. Quantitation of testosterone by radioimmunossay assay (RIA)

Testosterone was quantitated directly from the medium by RIA as described previously [29].

2.7. Measurement of ^{125}I-hCG binding to LH receptors

Quantitation of ^{125}I-hCG binding to LH receptors of cultured Leydig cells from neonatal rats was similar to the procedure described previously for cultured immature porcine Leydig cells [30] and which we have described for cultured Leydig cells from immature rats [31]. This method indirectly measures LH receptor number on Leydig cells [30].

2.8. Measurement of cellular cAMP levels

Cells were treated with increasing concentrations of OP and 10 mIU/mL hCG as described above. In addition, an identically-treated group of cells were cultured in the presence of 0.1 mM IBMX to inhibit phosphodiesterase activity. The final concentration of ethanol in these cells was 0.2% (IBMX was dissolved in ethanol). After 24 h of treatment, cells were washed with fresh culture media, then 0.5 mL of cold 70% ethanol was added to all the wells. Cells were disrupted using an Ultronics sonicator [32], and the content of each well was transferred to 12 × 75 mm borosilicate glass tubes. Tubes were centrifuged at ~1500 *g* to remove the precipitated protein. The supernatants were transferred to 1.7 mL Eppendorf tubes and dried using a Savant Speed Vac system. The dried residues were resuspended in cold 0.05 M acetate buffer (pH 5.8), and cAMP levels were quantitated following acetylation by RIA (Amersham cAMP kit, no. RPA509).

2.9. Statistical Analysis

Data were analyzed by analysis of variance (ANOVA). Differences among treatment groups were determined using Student-Newman-Keuls' test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Basal or hCG-stimulated testosterone production by cultured neonatal Leydig cells

Basal or 10 mIU/mL hCG-stimulated steroidogenic competence of cultured neonatal Leydig cells was evaluated by quantitating testosterone levels following 4, 28, and 76 h of culture/treatment. The testosterone level was 0.27 ± 0.02 ng/10^5 cells during the first 4 h of culture, increased to 0.55 ± 0.06 ng/10^5 cells during the next 24 h, then declined to 0.28 ± 0.03 ng/10^5 cells during the subsequent 48 h of culture in the absence of hCG (Fig. 1). In response to 10 mIU/mL hCG, the testosterone level was 0.81 ± 0.04 ng/10^5 cells during the first 4 h of exposure, increased to 32.4 ± 4.2 ng/10^5 cells during the next 24 h of exposure, and further increased to 79.2 ± 5.3 ng/10^5 cells during the subsequent 48 h of exposure. Steroidogenic competence

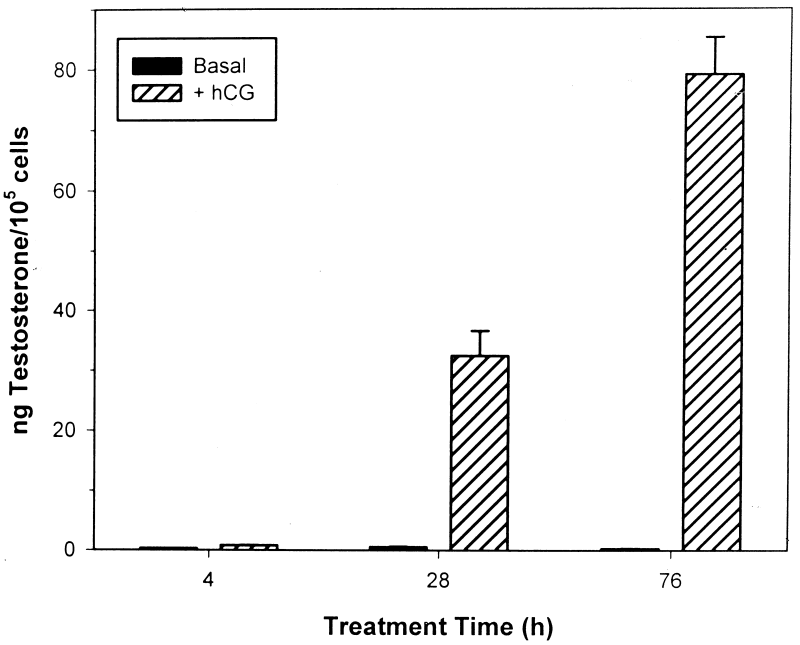

Fig. 1. Effect of time of culture or exposure on basal or hCG-stimulated testosterone formation. Neonatal Leydig cells were cultured for 2 d following plating before media change. Cells were left untreated or exposed to 10 mIU/mL hCG for 4 h. Media were collected, fresh media added and cells were left untreated or exposed to 10 mIU/mL hCG for 24 h. Media were collected, fresh media added, and cells were left untreated or exposed to 10 mIU/mL hCG for 48 h. Each value is the mean ± standard error of the mean (SEM) of 4 separate samples from a single experiment.

was maintained when the exposure period to hCG was extended for 2 to 4 additional days (data not shown). In contrast, cultured Leydig cells from adult rats progressively lose their steroidogenic competence in response to hCG during the first 3 d of culture [33]. These results demonstrate that cultured Leydig cells from neonatal rats are a sensitive model to evaluate either acute or chronic direct effects of environmental or occupational chemicals on fetal Leydig cell function.

3.2. Effect of acute exposure to octylphenol on basal or hCG-stimulated testosterone production

The testosterone level was 0.36 ± 0.03 ng/10^5 cells after incubation for 4 h in untreated cells (Fig. 2). Addition of increasing concentrations of OP (1 to 2000 nM) alone had no effect on basal testosterone levels. In response to 10 mIU/mL hCG, testosterone increased to 0.82 ± 0.04 ng/10^5 cells after exposure for 4 h. The inclusion of increasing concentrations of OP (1 to 500 nM) with hCG had no effect on testosterone levels, but 2000 nM OP + hCG decreased testosterone ~25% less than control ($P < 0.05$).

3.3. Effect of 24 h exposure to octylphenol on basal or hCG-stimulated testosterone formation

In the absence of hCG, the testosterone level was 0.56 ± 0.06 ng/10^5 cells after 24 h of culture (Fig. 3). The addition of increasing OP concentrations (1 to 2000 nM) had no effect on basal testosterone formation. In response to 10 mIU/mL hCG, testosterone increased to 10.78 ± 1.06 ng/10^5 cells. The addition of 1 and 10 nM OP (all OP concentrations were added ~30 min before treatment with hCG) increased hCG-stimulated testosterone to 13.80 ± 0.74 and 13.86 ± 0.80 ng/10^5 cells, respectively ($P < 0.05$ when compared to control). Testosterone declined progressively to 5.54 ± 0.58 ng/10^5 cell after addition of higher OP concentrations.

It should be noted that the increase in hCG-stimulated testosterone after exposure to lower OP concentrations for 24 h was variable (generally ~10 to 70% above control); however, the decline with higher OP concentrations was consistent (~40 to 80% less than control at the highest OP concentration).

3.4. Effect of octylphenol on 8-Br-cAMP-stimulated testosterone formation

To evaluate the site(s) of action of OP in altering testosterone formation, cultured neonatal Leydig cells were treated with increasing concentrations of OP (1 to 2000 nM) in the presence of 0.1 or 1.0 mM 8-Br-cAMP for 24 h. Testosterone level was 2.34 ± 0.27 ng/10^5 cells in response to 0.1 mM 8-Br-cAMP alone (Fig. 4). Testosterone levels were 3.11 ± 0.29 and 3.29 ± 0.54 ng/10^5 cells in response to treatment with 1 and 10 nM OP, respectively. These levels were not statistically higher than control. Addition of higher OP concentrations (100 to 2000 nM) progressively decreased 8-Br-cAMP-stimulated androgen levels, with testosterone declining to 0.84 ± 0.09 ng/10^5 cells at 2000 nM

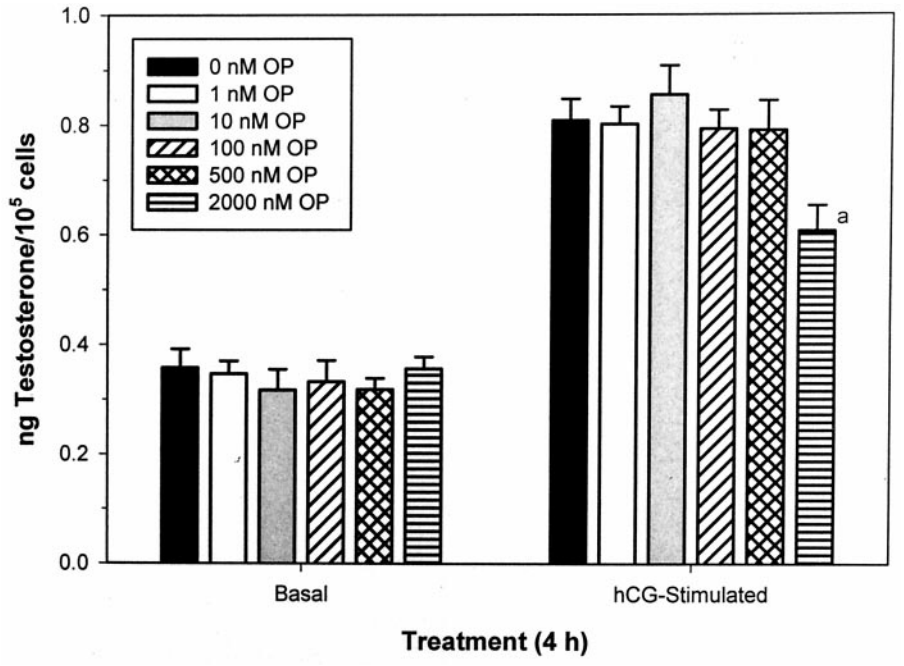

Fig. 2. Acute effects of octylphenol on basal or hCG-stimulated testosterone formation: 4 h exposure. Neonatal Leydig cells were cultured for 2 d following plating before media change and initiation of treatment. Octylphenol was dissolved in ethanol and the final concentration of ethanol in all treatment groups was 0.1%. hCG concentration was 10 mIU/mL. Following treatment for 4 h, media were collected for the quantitation of testosterone by RIA. Each treatment group represents the mean of 4 separate samples from a single experiment ±SEM. These results are representative of 3 separate experiments. $^{a}P < 0.05$ when compared to appropriate control.

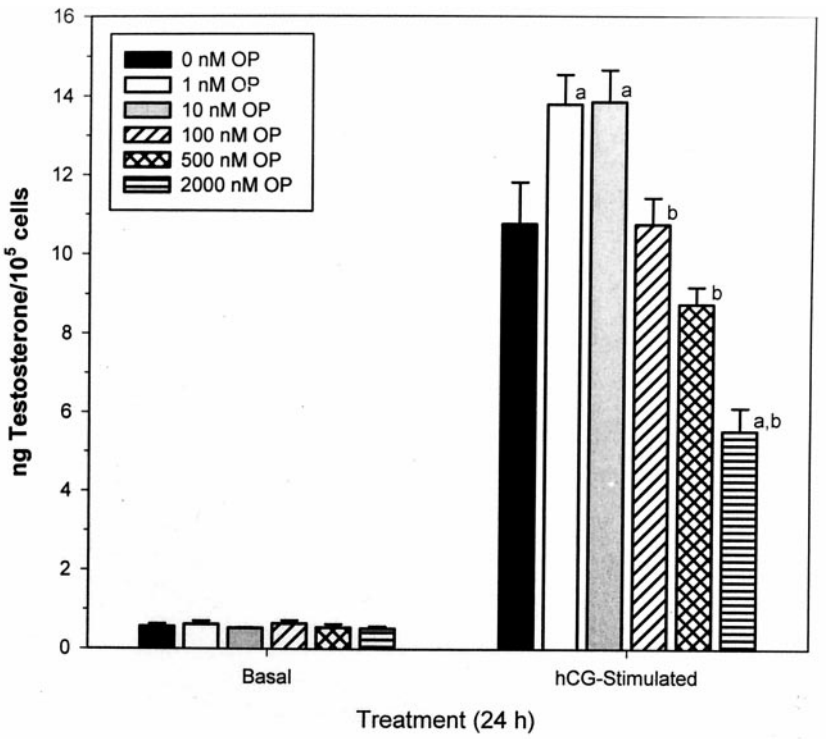

Fig. 3. Effects of octylphenol on basal or hCG-stimulated testosterone formation: 24 h exposure. Neonatal Leydig cells were cultured and treated as described in the legend for Fig. 1. Cells were treated for 24 h without or with 10 mIU/mL hCG. Media were collected for quantitation of testosterone by RIA. Each treatment group 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. $^{b}P < 0.05$ when compared to appropriate 10 nM OP-treated group.

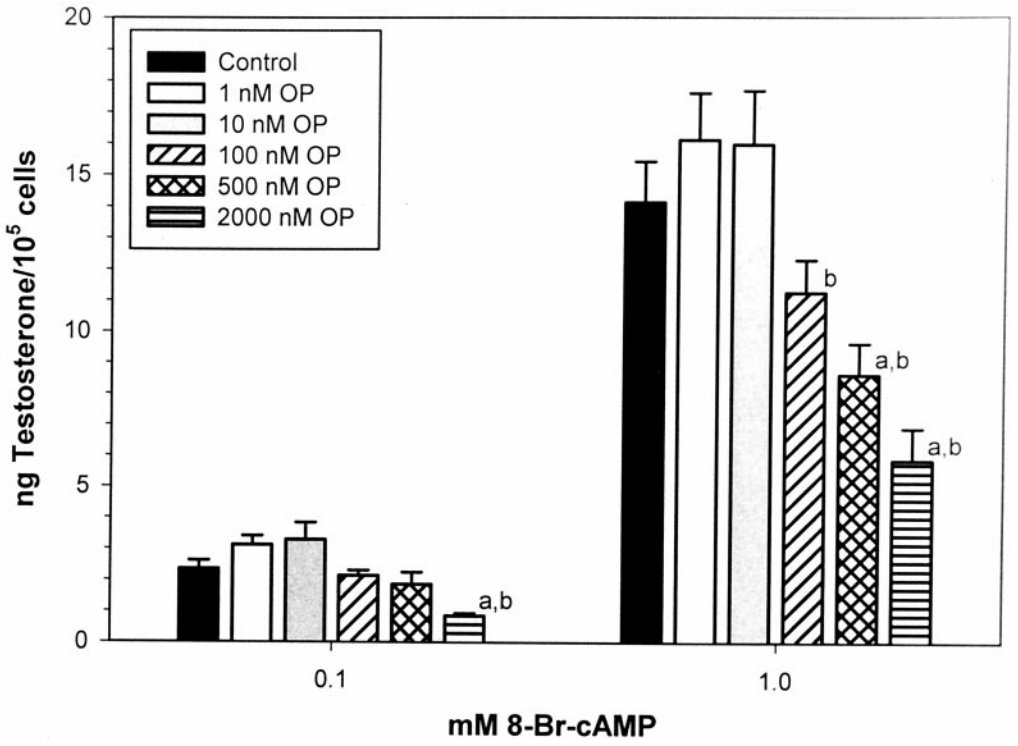

Fig. 4. Effects of octylphenol on 8-Br-cAMP-stimulated testosterone formation. Neonatal Leydig cells were cultured and processed as described in the legend for Fig. 3. Cells were treated with increasing concentrations of OP and 0.1 or 1.0 mM 8-Br-cAMP for 24 h, then media were collected for quantitation of testosterone by RIA. Each treatment group 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. [b]$P < 0.05$ when compared to appropriate 10 nM OP-treated group.

OP ($P < 0.05$ when compared to control or the 10 nM OP dose).

Whether OP (1 to 2000 nM) would elicit a similar biphasic testosterone response in the presence of a higher 8-Br-cAMP concentration (1.0 mM) was evaluated. In response to 1.0 mM 8-Br-cAMP alone, the testosterone level was 14.12 ± 1.30 ng/10^5 cells (Fig. 4, right panel). Testosterone levels were 16.11 ± 1.49 and 15.96 ± 1.72 ng/10^5 cells in response to 1 and 10 nM OP, respectively, and were not statistically higher than control. Exposure to higher OP concentrations (100 to 2000 nM) resulted in a progressive decline in testosterone levels to 5.84 ± 1.03 ng/10^5 cells at the highest concentration ($P < 0.05$ when compared to control or the 10 nM dose). These results suggest that the inhibitive effect of higher OP concentrations occur after the generation of cAMP; however, the increase in testosterone with exposure to lower OP concentration may occur both before and after the formation of cAMP.

3.5. Effect of octylphenol and hCG on cellular cAMP levels

Because the increase in testosterone levels in response to 1 and 10 nM OP was less in cells exposed to 0.1 or 1.0 mM 8-Br-cAMP than to 10 mIU/mL hCG, the possibility that lower OP concentrations increase cAMP formation/stability was examined. In response to exposure to increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h, intracellular cAMP levels were not affected by any of the OP concentrations added, in the absence or presence of 0.1 mM IBMX (data not shown).

3.6. Effect of octylphenol on ^{125}I-hCG binding to cultured neonatal Leydig cells

The possibility that the pattern of testosterone biosynthesis elicited by low and high concentrations of OP was due to changes in the number of LH receptors was evaluated by measuring the effects of OP on ^{125}I-hCG binding to cultured neonatal Leydig cells. Specific binding of ^{125}I-hCG to cultured neonatal Leydig cells treated with a low dose of hCG alone (1 mIU/mL) was 147 ± 23 cpm/well (Table 1). Treatment with 10 or 2000 nM OP and hCG for 24 h had no effect on ^{125}I-hCG binding to cultured cells. These results suggest that low and high OP concentrations do not alter LH receptor number in neonatal Leydig cells.

3.7. Effect of 17β-estradiol on hCG-stimulated testosterone production

Previous studies suggested that OP mimics the effects of estradiol in several cellular systems [11–13,17,19,20]. To evaluate whether estradiol produces similar effects on testosterone biosynthesis in cultured neonatal Leydig cells,

Table 1
Effects of 4-octylphenol and hCG on ^{125}I-hCG binding to cultured neonatal Leydig cells

Treatment		CPM/well ± SEM
nM OP	mIU/mL hCG	
0	1	147 ± 23
10	1	164 ± 16
2000	1	141 ± 13

The 2×10^5 neonatal Leydig cells were cultured for 2 d after plating. Fresh media were added, and cells were cultured in the presence of 1 mIU/mL hCG + 0, 10, or 2000 nM OP for 24 h. Cells were washed in glycine buffer, pH 3.0, to remove surface-bound unlabeled hCG, then incubated for 24 h with ^{125}I-hCG as described in the text. Separate samples in each treatment group received 25 IU/mL unlabeled hCG to estimate nonspecific binding, which was subtracted from total cpm to estimate specific binding (cpm/well). The results are the mean of three separate culture dishes from a single experiment and are representative of 3 separate experiments.

cells were exposed to increasing estradiol concentrations (1 to 1000 nM) and 10 mIU/mL hCG for 24 h. After treatment with hCG alone, the testosterone level was 15.22 ± 0.54 ng/10^5 cells (Fig. 5). Increasing concentrations of estradiol had no effect on testosterone biosynthesis. Similarly, exposure of cells for 2 or 4 additional days to these concentrations of estradiol and hCG had no effect on testosterone biosynthesis (data not shown).

3.8. Effect of ICI 182,780 on the effect of increasing concentrations of octylphenol on hCG-stimulated testosterone

The previous study demonstrated that the pattern of testosterone response to increasing estradiol and hCG differed from the response produced by treatment with increasing OP concentrations and hCG, suggesting that the actions of OP may not be mediated through the estrogen receptor (ER). This was evaluated further by treating cells with the pure estrogen antagonist, ICI 182,780 (ICI) [34], and with increasing OP concentrations and 10 mIU/mL hCG. ICI (10 or 100 nM) was added to the medium ~30 min prior to the addition of OP (1 to 2000 nM). With either concentration of ICI, increasing OP concentrations altered hCG-stimulated testosterone in a biphasic manner, with lower OP concentrations (1 and 10 nM) increasing testosterone levels (~60 to 70% above control), while higher concentrations of OP (100 to 2000 nM) decreased testosterone (~40 to 50% below control) (Fig. 6). These results suggest that the biphasic testosterone response to increasing OP concentrations is not mediated through the classic ER in neonatal rat Leydig cells.

3.9. Studies to determine whether the effects of octylphenol occur between the enzymatic steps that convert cholesterol to testosterone

To further localize possible site(s) of action of OP in eliciting the biphasic pattern of testosterone biosynthesis, cultured neonatal Leydig cells were treated with increasing OP concentrations (1 to 2000 nM) and 10 mIU/mL hCG for 24 h. Next, fresh media containing 1 μM 22 (R)-hydroxycholesterol, pregnenolone, progesterone, or androstenedione alone were added, and cells were incubated for an additional 4 h at 33°C. After addition of 1 μM androstenedione as substrate, the testosterone level was 31.85 ± 1.58 ng/10^5 cells in control cells (Fig. 7). Androstenedione conversion to testosterone was unaffected by any of the OP concentrations tested. The enzyme converting androstenedione to testosterone in rodent Leydig cells is the microsomal 17β-hydroxysteriod dehydrogenase type 3 (17β-HSD) [35]. These results suggest that the actions of OP are localized before the 17β-HSD step.

After addition of 1 μM progesterone as substrate to the control group (cells pretreated for 24 h with 10 mIU/mL hCG alone), the testosterone level was 15.92 ± 0.86 ng/10^5 cells (Fig. 7). Exposure of cells to increasing OP concentrations had no effect on testosterone levels. The conversion of progesterone to testosterone requires P450c17 activity (which contains both 17α-hydroxylase and C17–20-lyase activities, and which converts progesterone to 17α-hydroxyprogesterone and androstenedione, respectively) and 17β-HSD activity. These results suggest that OP effects are localized prior to the P450c17 step.

The enzyme converting pregnenolone to progesterone in rat Leydig cells is 3β-hydroxysteroid dehydrogenase-isomerase type I (3β-HSD), while the enzyme converting cholesterol to pregnenolone is the mitochondrial P450 side-chain cleavage activity (P450scc). To determine whether OP alters these enzymic steps, fresh media containing 1 μM pregnenolone or 22(R)-hydroxycholesterol, respectively, were added for 4 h at 33°C to cells pretreated for 24 h with increasing OP concentrations and 10 mIU/mL hCG. In the control groups, testosterone levels were 4.08 ± 0.31 and 3.64 ± 0.34 ng/10^5 cells, respectively (Fig. 7). The conversion of either substrate to testosterone was not affected by prior exposure to increasing OP concentrations, suggesting that the primary actions of OP occur before the mitochondrial P450scc step.

3.10. Effect of concommitant treatment with antioxidants on biphasic response to increasing octylphenol concentrations and hCG

The possibility that the biphasic testosterone response to OP was due to its anti- or pro-oxidant properties at low and high concentrations, respectively, was examined. In cells exposed concomitantly for 24 h to 100 μM α-tocopherol and 10 mIU/mL hCG alone (control group), the testosterone level was 25.60 ± 1.66 ng/10^5 cells (Fig. 8). Testosterone levels were 30.21 ± 2.80 and 33.25 ± 4.24 ng/10^5 cells in response to 1 and 10 nM OP, respectively, which were not statistically higher than control. However, testosterone progressively declined after exposure to higher OP concentrations, and was 14.38 ± 1.10 ng/10^5 cells after addition of

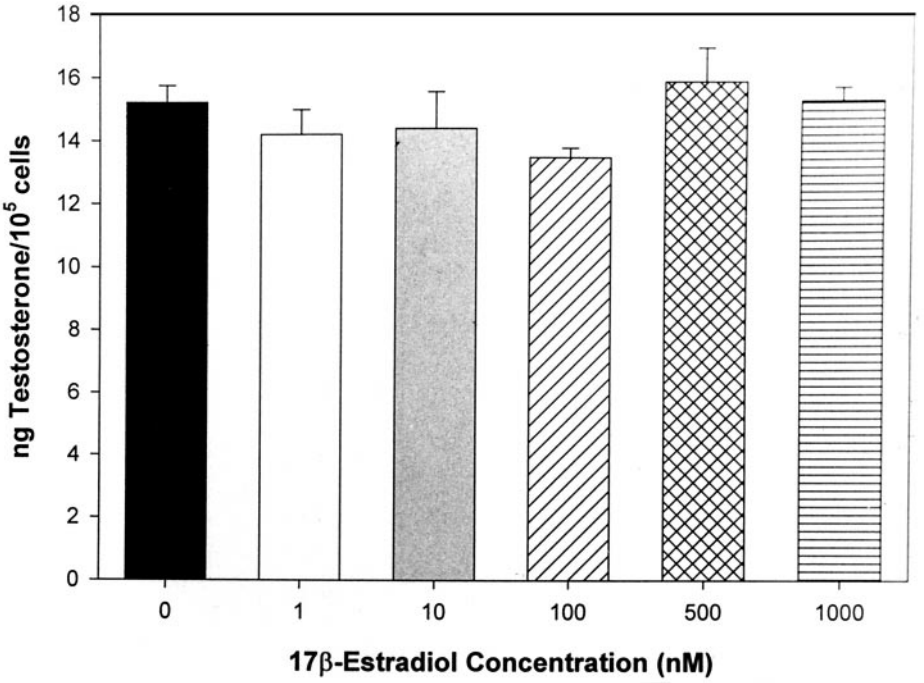

Fig. 5. Effects of 17β-estradiol on hCG-stimulated testosterone formation. Neonatal Leydig cells were cultured as described in the legend for Fig. 3. Cells were treated with increasing concentrations of estradiol (0, 1 to 1000 nM) and 10 mIU/mL hCG for 24 h. All treatment groups received 0.1% ethanol (diluent for estradiol). Media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of 4 separate samples from a single experiment, and these results are representative of at least 3 separate experiments.

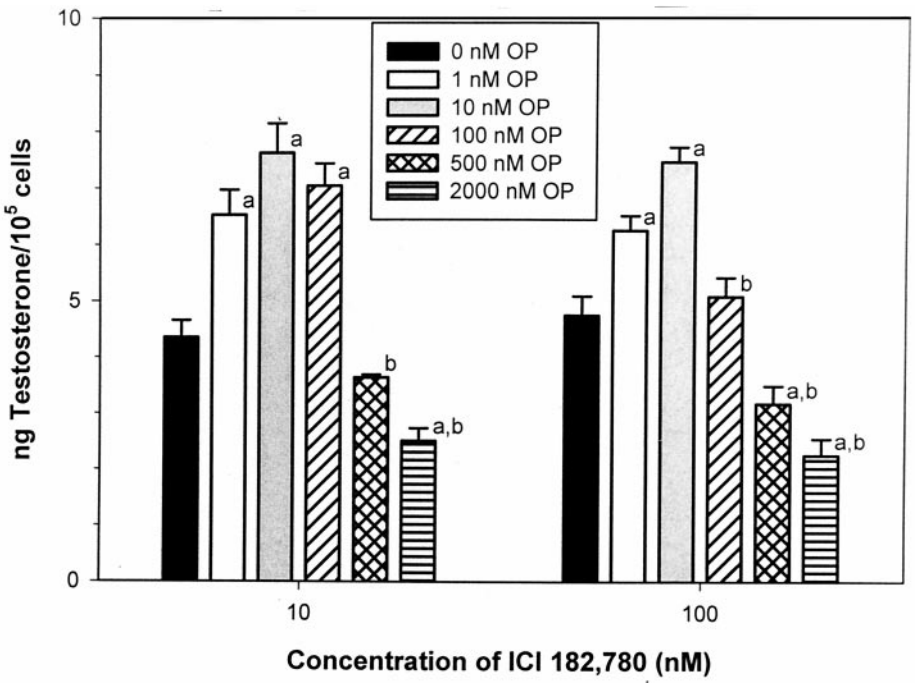

Fig. 6. Effects of ICI 182,780 on testosterone response to increasing concentrations of octylphenol and to hCG. Neonatal Leydig cells were cultured as described in the legend for Fig. 3. Cells were treated with 10 or 100 nM ICI 30 min prior to exposure to increasing concentrations of OP and 10 mIU/mL hCG. Media were collected 24 h following treatment for quantitation of testosterone by RIA. Each treatment group 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. $^{b}P < 0.05$ when compared to appropriate 10 nM OP-treated group.

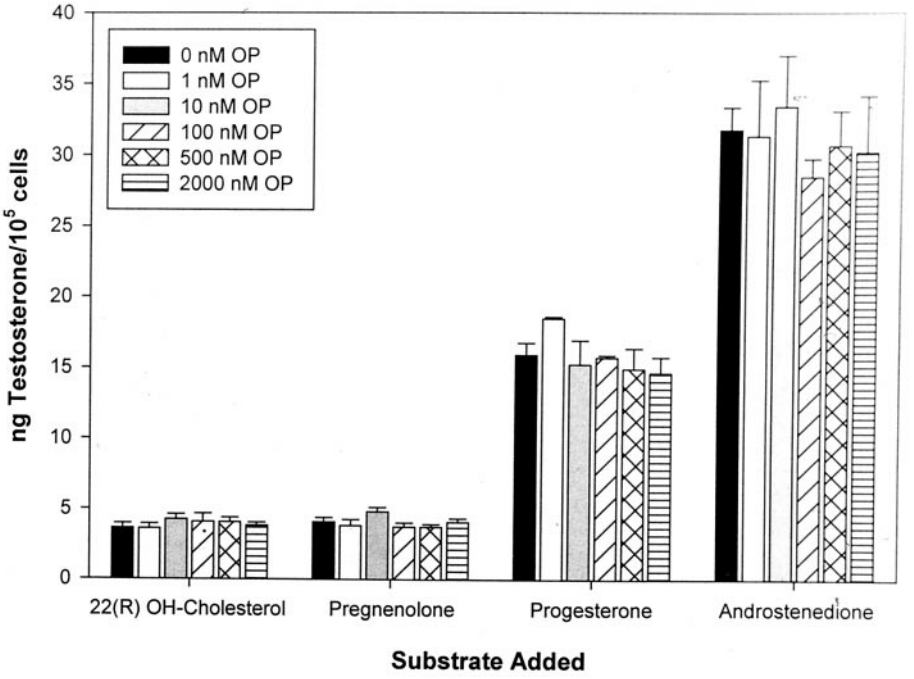

Fig. 7. Precursor substrate conversion to testosterone following exposure to increasing concentrations of octylphenol and to hCG. Neonatal Leydig cells were cultured as described in the legend for Fig. 3. Cells were treated with increasing concentrations of OP and 10 mIU/mL hCG for 24 h. Next, fresh media containing 1 μM 22 (R)-hydroxycholesterol, pregnenolone, progesterone, or androstenedione were added to all treatment groups, and cells were incubated for 4 h at 33°C. Media were collected for quantitation of testosterone by RIA. Each treatment group represents the mean ± SEM of 4 separate samples from a single experiment. These results are representative of at least 3 separate experiments.

2000 nM OP ($P < 0.05$ when compared to appropriate control or 10 nM dose). The testosterone level of control cells exposed concomitantly to hCG and 100 μM butylated hydroxyanisole (BHA) was 13.33 ± 1.02 ng/10^5 cells (Fig. 8, middle panel). In response to 1 and 10 nM OP, testosterone levels were 14.21 ± 1.44 and 18.20 ± 1.33 ng/10^5

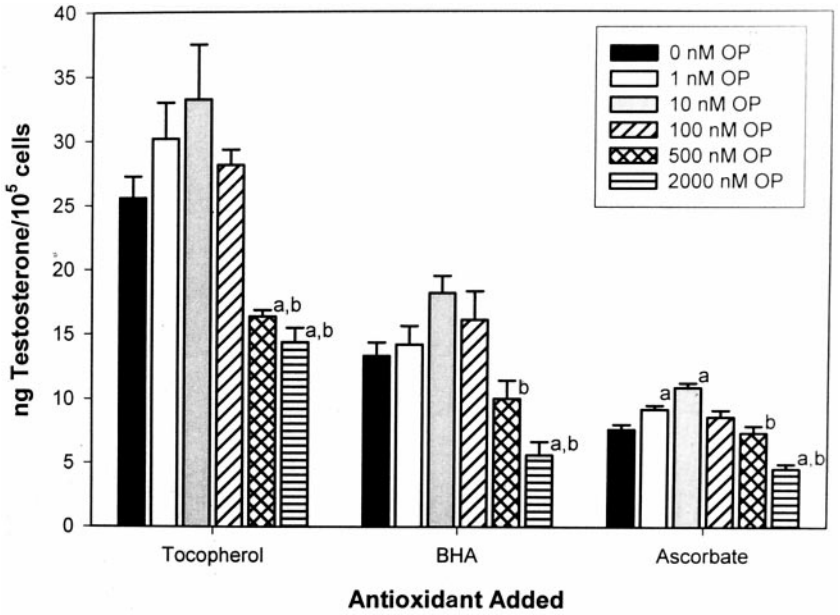

Fig. 8. Effects of concommitant treatments with antioxidants on testosterone response to increasing concentrations of octylphenol and to hCG. Neonatal Leydig cells were cultured as described in the legend for Fig. 6. Cells were exposed to 100 μM α-tocopherol, BHA, or ascorbate approximately 30 min prior to the addition of increasing OP concentrations and 10 mIU/mL hCG. Media were collected 24 h following treatment for quantitation of testosterone by RIA. Each treatment group 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 group. [b]$P < 0.05$ when compared to appropriate 10 nM OP-treated group.

cells, respectively, which were not statistically higher than control. In response to higher OP concentrations, testosterone levels progressively declined to 5.62 ± 1.05 ng/10^5 cells at the 2000 nM dose ($P < 0.05$ when compared to appropriate control or 10 nM dose). In hCG-treated control cells concomitantly exposed to 100 μM ascorbate, the testosterone level was 7.63 ± 0.38 ng/10^5 cells (Fig. 8, right panel). In response to exposure to 1 and 10 nM OP, testosterone levels increased to 9.19 ± 0.30 and 10.88 ± 0.36 ng/10^5 cells, respectively (both $P < 0.05$ when compared to appropriate control). Exposure to higher OP concentrations progressively decreased testosterone levels to 4.57 ± 0.35 ng/10^5 cells at the 2000 nM dose ($P < 0.05$ when compared to appropriate control or 10 nM dose). The differences in testosterone levels among the hCG-treated controls exposed to 100 μM α-tocopherol, BHA or ascorbate were not due to the effect of the antioxidant, but to the use of different cellular preparations. In preliminary studies, testosterone levels were unaltered by these concentrations of antioxidants. These results suggest that OP is not acting as a pseudosubstrate/prooxidant in eliciting its inhibitive effects at higher concentrations. The fact that we observed varying degrees of enhanced testosterone levels in the presence of 1 and 10 nM OP + hCG and each antioxidant (although the increases in the presence of α-tocopherol or BHA lacked statistical significance compared to control), suggests that a possible antioxidant property of lower OP concentrations is not the primary mechanism to explain the increase in androgen.

4. Discussion

The results of the present studies suggest that direct exposure of cultured neonatal rat Leydig cells to increasing concentrations of OP (1 to 2000 nM) has a biphasic effect on hCG-stimulated testosterone formation. Lower doses of OP (1 and 10 nM) generally enhance testosterone formation (~10 to 70% above control), whereas higher concentrations (100 to 2000 nM) progressively decrease testosterone levels to ~40 to 80% less than control at the highest OP concentration. Although OP has been reported to behave as a weak estrogen in several cellular systems, these effects of OP are not mimicked by 17β-estradiol, nor do they appear to be mediated through activation of the classic estrogen receptor (ER). The primary site(s) of action of OP in eliciting this biphasic testosterone response seem to be localized after the generation of cAMP and before the side-chain cleavage of cholesterol.

We are unaware of any previous studies reporting an increase in testosterone formation after treatment of neonatal rat Leydig cells with low concentrations of OP. Previous in vivo maternal exposure of pregnant rats on Days 11.5 and 15.5 postcoitum (p.c.) and assay of fetal testes on Day 17.5 p.c. (total exposure of 6 d) was reported to reduce P450c17 activity and protein levels as detected by immunohistochemistry [17] and the expression of steroidogenic factor 1 (SF-1) [19], a transcription factor involved in the differentiation of steroidogenic organs. Under the present in vitro conditions, we did not detect a direct effect of OP on P450c17 activity in cultured neonatal rat Leydig cells. In another in vivo study, chronic administration of OP to adult rats was reported to reduce serum testosterone concentrations, although serum LH and FSH levels also were depressed [20]. These inhibiting effects of OP in vivo probably were achieved at higher dosages of OP, although actual circulating levels of OP in these animals were not reported. Whether lower dosages of OP administered in vivo to fetal/neonatal rats would augment testosterone production by Leydig cells is not known. Furthermore, even if increases in testosterone production were observed under these conditions, whether these changes would be sufficient to alter normal reproductive development is uncertain. The mechanism(s) by which lower OP concentrations enhance testosterone production is not known. Because lower doses of OP had no effect on cellular cAMP levels in the absence or presence of IBMX in the present study, the increase in testosterone does not seem to be mediated by an increase in cAMP formation or stability.

Previous studies relating to direct estrogen effects on the testis are conflicting. Two studies reported that only high estradiol concentrations (>180 μM) inhibited hCG-stimulated testosterone formation after acute exposure of testicular tissue from adult rats [36,37]. However, another study reported that exposure of cultured Leydig cells from adult rats for 24 h with 0.4 to 400 nM estradiol caused a dose-dependent decrease in hCG-stimulated testosterone production and that the main site of action was the microsonal P450c17 step [38]. In cultured Leydig cells from fetal rats (Day 21 of gestation), treatment with 1.1 μM estradiol for 2 d had no effect on LH-stimulated testosterone production; however, treatment for one additional day resulted in a decline in testosterone formation, suggesting that fetal Leydig cells acquired a sensitivity to estradiol following additional in vitro exposure that was mediated through the ER [39]. Although no direct effect of estradiol (1 to 1000 nM) on hCG-stimulated testosterone formation was observed after 24 h of exposure in the present study, treatments up to 4 d with estradiol similarly were without effect (data not shown). Age differences of the animals from which Leydig cells were collected could explain these response differences to estradiol in the previous and present studies; however, Leydig cells present during the neonatal period represent fetal Leydig cells [25], and would be expected to respond similarly to estradiol.

The fact that we observed a biphasic testosterone response to increasing concentrations of OP in the presence of hCG, but no effect of estradiol in the present study suggests that OP has effects on neonatal Leydig cells that are intrinsically different from the native steroid. The inability of the pure estrogen antagonist, ICI 182,780 [34], to alter this biphasic response in the present studies suggest that these

effects of OP are not mediated through the ER. Both ERα and ERβ subtypes have been identified in fetal/neonatal Leydig cells by immunohistochemistry [40,41]; however, it has not been established whether they are functional at this stage of development. Although some actions of OP in neonatal Leydig cells may be mediated through ERα and/or ERβ, OP appears to alter testosterone biosynthesis by another pathway/mechanism.

Because the present studies suggested that the pattern of testosterone biosynthesis in response to increasing concentrations of OP and to hCG was not mediated through the ER, the possibility that this could be explained by its actions as an anti- or pro-oxidant was examined. Two of the steroidogenic enzymes involved in the conversion of cholesterol to testosterone in Leydig cells are cytochrome P450 enzymes (P450scc and P450c17). They use molecular oxygen and electrons donated from NADPH for hydroxylation of the substrate. During normal steroidogenesis, reactive oxygen species (superoxide and/or hydroxy radical) can be produced by electron leakage outside the electron transfer chains [42,43], and these free radicals can initiate lipid peroxidation, which can inactivate P450 enzymes [44]. Antioxidants, such as α-tocopherol, ascorbate, BHA, or dimethyl sulfoxide, have been reported to protect P450 enzymes from lipid peroxidative damage [45]. Normal products of steroidogenesis as well as exogenous chemicals can act as pseudosubstrates. These products can bind to the substrate-binding site of P450 enzymes, but cannot be hydroxylated [45]. This phenomenon can lead to electron leakage and formation of free radicals [45]. Thus, compounds that have antioxidant properties or act as a pseudosubstrate could enhance or inhibit steriod production, respectively. Because the increase in testosterone with lower OP concentrations was attenuated with concommitant exposure to α-tocopherol or BHA, it does not seem that this increase is due primarily to its antioxidant properties. Furthermore, because all three antioxidants did not alter the pattern of testosterone decline with higher OP concentrations, it does not seem that this inhibition is due to OP acting as a pseudosubstrate (pro-oxidant).

Although previous studies have suggested that OP is estrogenic and mediates its effects through the ER in several cellular systems [11–13], the present findings demonstrate that some actions of OP may be mediated through a separate pathway in neonatal rat Leydig cells. In ERα knockout mice (ERKO), both 4-hydroxyestradiol and the insecticide chlordecone (Kepone) were reported to induce uterine lactoferrin expression by a pathway that did not involve nuclear ERα or ERβ [46]. Another study found that some xenoestrogens enhanced the expression of estrogen-responsive genes in the uterus of CD-1 mice by mechanisms that did not involve the classical ERs [47]. Other pathways for estrogen-mediated actions have been reported. For example, rapid (minutes) membrane-associated activation of mitogen-activated protein kinase was reported after treatment of a human neuroblastoma cell line with membrane impermeable estradiol-bovine albumin conjugate (E_2-BSA) [48]. Furthermore, this activation was unaffected by ICI 182,780, suggesting that this effect of E_2-BSA is not mediated through binding to the classic ERα or ERβ. In addition, estradiol has been reported to interact with other signaling pathways [49,50], and various factors have been shown to modulate androgen biosynthesis by Leydig cells. For example, both insulin and insulin-like growth factor I (IGF-I) have been shown to enhance testosterone biosynthesis by rat Leydig cells [51], whereas epidermal growth factor [52], fibroblast growth factor II [53], tumor necrosis factor-α [54], and interleukin-1 [55] were reported to inhibit testosterone formation by rodent Leydig cells. Thus, activation or interference of these other signaling pathways by OP could either enhance or inhibit testosterone formation by neonatal Leydig cells. The current studies focused on the effects of OP on neonatal (fetal) Leydig cells. It will be interesting to determine whether the maturational status of the Leydig cell influences its response to estradiol, OP, or similar chemicals.

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