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Octylphenol inhibits testosterone biosynthesis by cultured precursor and immature Leydig cells from rat testes

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 2015, 1095 Willowdale Road, Morgantown, WV 26505-2888, USA

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Abstract

4-tert-octyphenol (OP) is a surfactant additive widely used in the manufacture of a variety of detergents and plastic products. OP has been reported to mimic the actions of estrogen in many cellular systems. The present studies evaluated the direct effects of OP on human chorionic gonadotropin (hCG)-stimulated testosterone biosynthesis by cultured precursor and immature Leydig cells from 23-day old (prepubertal) rats. Exposure to increasing OP concentrations (1 to 2000 nM) progressively decreased hCG-stimulated testosterone formation in both precursor and immature Leydig cells at higher OP concentrations (100 or 500 to 2000 nM). Testosterone levels were reduced ~ 30 to 70% below control at the highest concentration in both cell types. Similar reductions in testosterone associated with OP exposure were observed in cells stimulated with 1 mM 8-Br-cAMP, suggesting that the main actions of OP occur after the generation of cAMP. Increasing concentrations of 17β-estradiol (1 to 1000 nM) had no effect on hCG-stimulated testosterone formation in both precursor and immature Leydig cells and the inclusion of 100 nM ICI 182,780, a pure estrogen antagonist, in precursor and immature Leydig cells exposed to OP and hCG, did not alter the inhibition by higher OP concentrations of testosterone formation in both cell types. These results suggest that OP is a hormonally active agent, but that some of its actions are distinct from those of 17β-estradiol and are not mediated through the estrogen receptor α or β pathway. To further localize the potential site(s) of action of OP, cultured precursor and immature Leydig cells were exposed to increasing concentrations of OP and hCG for 24 h. Next, fresh media containing 1 μM 22(R)-hydroxycholesterol, 1 μM pregnenolone, 1 μM progesterone, or 1 μM androstenedione was added, and the conversion of each substrate to testosterone was determined after incubation for 4 h. The conversion of androstenedione to testosterone was unaffected by exposure to OP, suggesting that the 17β-hydroxysteroid dehydrogenase step is not inhibited. However, the conversion of 22(R)-hydroxycholesterol, pregnenolone and progesterone all were inhibited by prior exposure to OP and hCG. This finding suggests that the 17α-hydroxylase/c17–20-lyase step, which converts progesterone to androstenedione, is inhibited by OP, and that the cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase -isomerase steps, which convert cholesterol to pregnenolone and pregnenolone to progesterone, respectively, are other potential sites of OP action. Because concomitant exposure to the antioxidants α-tocopherol or ascorbate did not alter the inhibition of testosterone formation by higher OP concentrations, it does not appear that OP is acting as a pseudosubstrate for the generation of free radicals, which can damage P450 enzymes.

Keywords: Leydig cell precursors; Immature Leydig cells; Testosterone; Octylphenol

1. Introduction

It has been proposed that environmental/occupational exposure to endocrine-disrupting chemicals adversely affects the reproductive system of humans and various wildlife species [1]. Potential changes occurring in males over the past 40–50 years include: 1) an international 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 in utero exposure to estrogen-mimicking chemicals [5], this idea is not totally accepted [6], and it has not been established whether such changes have any impact on male fertility [7]. Despite the controversy, there is general agreement on the need of additional in vivo and in vitro studies to establish if a connection exists between exposure to these chemicals and an adverse effect on male reproductive function [8].

* 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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Alkylphenol ethoxylates (APEs) are a class of nonionic surfactants widely used in the production of agricultural, industrial, and household detergents, and in the formulation of paints, herbicides, pesticides, and plastics [9]. The primary alkyl groups are composed of branched nonyl or octyl chains positioned opposite the para-substituted ethoxylate chain, which is composed of 1 to 100 repeating ethylene oxide units [10]. 4-Tert-octylphenol (octylphenol; OP) and 4-nonylphenol are degradation products of APEs that are environmentally persistent [11]. With respect to estrogen-like effects, OP stimulated vitellogenin production in trout cultured hepatocytes [10], growth of a MCF-7 human breast cancer cell line [10], and estrogen-dependent β-galactosidase activity in a yeast estrogen screen (YES) assay [12]. However, OP was approximately 1000 times less potent than 17β-estradiol or the synthetic estrogen, diethylstilbestrol (DES).

It has been reported that inappropriate exposure of males to estrogenic chemicals during gestation can adversely affect reproductive development. Thus, male offspring of mothers treated during the 1950s and 1960s with DES to prevent complications of pregnancy were reported to have a higher incidence of cryptorchidism, testicular hypoplasia and semen abnormalities [13]. In mice, male offspring of pregnant animals treated with DES were reported to have a higher incidence of sterility [14] and cryptorchidism [15]. In pregnant rats, exposure to DES or OP was reported to reduce the amount of the steroidogenic enzyme P450 17α-hydroxylase/c17-20-lyase (P450c17) [16], and the expression of steroidogenic factor-1 (SF-1/Ad4BP), a transcription factor involved in the development of the adrenals and gonads, in fetal testis [17]. Chronic treatment of adult males with OP or estradiol valerate was reported to reduce testis size and sperm numbers [18]. Although these in vivo studies did not clarify whether the actions of 17β-estradiol, DES, or OP were direct or indirect effects on the testis, a recent study reported that OP inhibited progesterone biosynthesis by cultured mouse Leydig tumor cells (mLTC-1) [19]. In addition, we reported recently that OP had a biphasic effect on hCG-stimulated testosterone formation by cultured Leydig cells from neonatal rats, with lower concentrations (1 and 10 nM) actually enhancing testosterone levels, followed by a progressive reduction in testosterone at higher levels (100 to 2000 nM) [20]. In contrast to these reproductive effects of OP in various animal models, a recent multigeneration study reported that exposure to OP in the feed had limited effects on the reproductive system of Sprague–Dawley rats [21].

Leydig cells in rodents exhibit several distinct maturational stages. In rats, fetal Leydig cells appear and initiate testosterone secretion on approximately Day 15 of gestation [22]. The fetal Leydig cells persist for about 2 to 3 weeks after birth, and progressively regress thereafter [23]. Beginning around 14 days after birth and continuing for about the next 2 weeks, a second generation of Leydig cells appear, mainly through differentiation from mesenchymal precursor cells localized within the interstitium [24]. This process appears to be regulated mainly by luteinizing hormone (LH) [25,26]. Although immature rat Leydig cells actively synthesize testosterone, it does not accumulate and is not secreted because high 5α-reductase activity rapidly converts this androgen to 5α-reduced metabolites, which are the main androgens secreted [27]. Testosterone levels progressively increase after about 40 days of age because of the progressive decline in 5α-reductase activity [28]. Thus, the adult stage of Leydig cells is established and is represented by low 5α-reductase activity and the secretion of testosterone as the predominant androgen, observed after about 60 days of age [29].

We are unaware of any studies that have examined the direct effects of OP on testosterone biosynthesis by precursor and immature Leydig cells. Therefore, the present studies evaluated whether varying concentrations of OP altered human chorionic gonadotropin- (hCG) stimulated testosterone biosynthesis by rat cultured precursor and immature Leydig cells. Because testosterone plays such a critical role in the development of secondary sexual characteristics and the initiation of spermatogenesis during pubertal maturation, reductions in testosterone due to exposure to OP and similar xenobiotics have the potential to adversely affect normal sexual development in humans.

2. Materials and methods

2.1. Animals

Immature male Sprague–Dawley rats (17 days of age) with nursing mothers were purchased from Hilltop Lab Animals, Inc., Scottdale, PA, USA. Animals were weaned at 21 days of age and housed in shoebox cages (four per cage). They were 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 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, 22(R)-hydroxycholesterol, 5-pregnen-3β-ol-20-one (pregnenolone), 4-pregnen-3,20,-dione (progesterone), 4-androsten-3,17-dione (androstenedione), diethylstilbestrol (DES), α-tocopherol, L-ascorbic acid, 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) (BSA, clinical reagent grade) and Ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals, Inc., Costa Mesa, CA.

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-*N*′-2-ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY, USA. [1,2,6,7-^{3}H (N)]-Testosterone (specific activity ~ 100 Ci/mmol) and [^{125}I]-human chorionic gonadotropin (^{125}I-hCG, specific activity ~ 50 μCi/mg) were from NEN Life Science Products, Boston, MA, USA. The cAMP RIA Kit was from Amersham, Arlington Heights, IL, USA. Tissue culture plates (24-well, 1.6 cm diameter) were from Corning/Costar, Cambridge, 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) was from Aldrich Chemical Co., Milwaukee, WI, USA, and its structure has been presented previously [10]. Percoll was from Pharmacia, Piscataway, NJ, USA. Human chorionic gonadotropin (hCG, CR-127, specific activity 14 900 IU/mg) was a gift from NIDDK, Bethesda, MD, USA. Sodium 4-aza-4-methyl-3-oxo-5α-pregnan-20(S)-carboxylate (4-MOPC), a 5α-reductase inhibitor, was a gift from Merck, Sharpe and Dohme, Rahway, NJ, USA.

2.3. Isolation of precursor and immature Leydig cells

Animals were 23-day old when sacrificed by intraperitoneal (i.p.) injection of pentobarbitol. To obtain sufficient cells for culture, testes from 30 to 50 animals were removed, 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. Dispersed interstitial cells were elutriated using a Beckman elutriation apparatus as described previously for adult rats [30], but modified for interstitial cells from immature rats [31]. Cells collected after elutriation were layered over a 60% Percoll gradient and centrifuged at ~ 25 000 × *g* for 1 h. Precursor cells localized between densities of ~ 1.05 to 1.07 g/mL whereas immature Leydig cells localized between densities of ~ 1.07 and 1.09 g/mL [32]. For each study, a minimum of three separate pools of cells was evaluated. In previous studies the immature Leydig cell fraction localizing at the higher density region of Percoll gradients was reported to contain > 93% Leydig cells when isolated from 25- [31] and 35-day old animals [33] based on dark staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. Cells obtained from 23-day old animals and isolated from the same region of Percoll-density gradients contained ~ 90% Leydig cells. Cells obtained from 21-day old animals and localizing in the lower density fraction of Percoll gradients were reported to contain ~ 90% precursor cells, which stained lightly for 3β-HSD [30,34]. However, in the present study, using cells from 23-day old animals, we estimated that approximately 80% of the cells localizing in the lower density region represented precursor cells (based on light staining for 3β-HSD). It seems that higher percentage yields of precursor and immature Leydig cells can be obtained by using slightly younger (21-day old) and older (25- to 35-day old) immature rats, respectively. We selected 23-day old rats because both cell types can be isolated from these animals and the purity of the cells is only slightly lower for both fractions.

2.4. Culture of precursor and immature Leydig cells

Precursor and immature 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 [20]. One mL of cells (10^5/mL) was plated into each 1.6 cm diameter well of a 24-well Costar culture plate and cultured 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 ~ 20 h after plating and treatments were initiated. Cells were exposed to various treatments for 4 or 24 h, but up to 5 days depending on the experiment, with media change and retreatment every other day of culture for studies extending beyond 24 h.

2.5. Treatment of cells

OP and 17β-estradiol were dissolved in ethanol. The final ethanol concentration in all treatment groups (including controls) was 0.1%. This concentration did not affect testosterone biosynthesis or cell viability. Furthermore, the concentrations of chemicals used in these experiments (0,1,10,100,500, and 2000 nM for OP and 0,1,10,100,500, and 1000 nM for 17β-estradiol) did not affect cell viability based on cell morphology, attachment to culture plates, and trypan blue exclusion criteria. In addition, both precursor and immature Leydig cell cultures received 1 μM 4-MOPC to block 5α-reductase activity and thereby inhibit the metabolism of testosterone to dihydrotestosterone (DHT) and hydroxylated metabolites of DHT (5α-androstan-3α, 17β-diol and 5α-androstan-3β,17β-diol) [32]. In preliminary studies we observed that OP had no effect on 5α-reductase activity in these cells.

2.6. Quantitation of testosterone production and of ^{125}I-hCG binding to LH receptors

Testosterone was quantitated directly from the culture medium by radioimmunoassay (RIA) as described previously [35]. Quantitation of ^{125}I-hCG binding to LH receptors of cultured precursor and immature Leydig cells was similar to the procedure described for cultured immature porcine Leydig cells [36], which we have modified for

immature rat Leydig cells [31]. This method indirectly measures LH receptor number on Leydig cells [36].

2.7. Measurement of cellular cAMP

Cultured precursor and immature Leydig cells were treated with increasing concentrations of OP and 10 mIU/mL hCG for 4 or 24 h in the absence or presence of 0.1 mM IBMX (to inhibit phosphodiesterase activity). The final ethanol concentration in these cells was 0.2% (IBMX was dissolved in ethanol). After treatment, cells were washed with fresh media, and 0.5-mL cold 70% ethanol was added to each well. Cells were disrupted using an Ultronics sonicator [37], and the content of each well was transferred to a 12 × 75 mm borosilicate glass tube. Samples were centrifuged to remove precipitated protein, and the supernatants were transferred to 1.7-mL Eppendorf tubes. The supernatants were evaporated 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 after acetylation by RIA (Amersham cAMP Kit, no. RPA509).

2.8. Statistical analysis

Data were analyzed by ANOVA. Differences among treatments were determined using Student-Newman–Keuls' test. A P value <0.05 was considered statistically significant.

3. Results

3.1. Effect of octylphenol on hCG-stimulated testosterone

Testosterone level in cultured precursor cells exposed to 10 mIU/mL hCG for 4 h was 0.42 ± 0.06 ng/10^5 cells (Fig. 1A). Exposure to increasing OP concentrations (1 to 2000 nM) had no effect on hCG-stimulated testosterone. The testosterone level in cultured immature Leydig cells exposed for 4 h to 10 mIU/mL hCG was 2.54 ± 0.12 ng/10^5 cells. Exposure to increasing OP concentrations (1 to 2000 nM) had no effect on hCG-stimulated testosterone formation (Fig. 1A).

Testosterone level in cultured precursor cells exposed to 10 mIU/mL hCG for 24 h was 11.97 ± 1.06 ng/10^5 cells (Fig. 1B). Exposure to 1 or 10 nM OP had no effect on testosterone levels; however, levels declined progressively to 9.05 ± 0.19, 6.47 ± 0.49, and 5.33 ± 0.24 ng/10^5 cells when exposed to 100, 500, and 2000 nM OP, respectively ($P < 0.05$ for each compared to control). The testosterone level of cultured immature Leydig cells exposed for 24 h to 10 mIU/mL hCG was 33.37 ± 1.80 ng/10^5 cells (Fig. 1B). OP concentrations of 1 to 100 nM did not alter testosterone levels; however, 500 and 2000 nM OP reduced testosterone to 20.44 ± 4.85 and 15.73 ± 1.15 ng/10^5 cells, respectively ($P < 0.05$ when both compared to control). Adding fresh media alone for 24 to 72 h to previously exposed cells, followed by treatment with hCG and 4-MOPC alone, allowed testosterone levels of OP-exposed cells to recover to control levels (data not shown), suggesting that higher OP concentrations did not affect cell viability.

There was variability in the level of testosterone produced after exposure to hCG for 24 h, the dose of OP at which significant declines in testosterone formation was first detected, and the degree of the net decline in androgen formation at the highest OP concentration evaluated in both precursor and immature Leydig cells. Testosterone levels produced by precursor cells ranged from 2.59 to 11.97 ng/10^5 cells, whereas in immature Leydig cells, they ranged from 5.68 to 33.37 ng/10^5 cells. In general, the ratio of testosterone produced by immature Leydig cells to precursor cells was from 3 to 6. With respect to sensitivity, significant declines in testosterone were observed at both 500 and 2000 nM OP in both precursor and immature Leydig cells; however, in some cases significant declines were detected at 100 nM OP. Maximal declines in testosterone at the highest OP concentration evaluated varied from ~ 30 to 70% below control for both precursor and immature Leydig cells.

3.2. Effect of octylphenol on 8-Br-cAMP-stimulated testosterone

Because the primary action of hCG in stimulating testosterone biosynthesis in Leydig cells is mediated through a rise in intracellular cAMP, we evaluated whether the effects of OP in precursor and immature Leydig cells were localized before or after the formation of cAMP. In response to 1 mM 8-Br-cAMP alone for 24 h, the testosterone level was 5.07 ± 0.36 ng/10^5 in precursor cells (Fig. 2). Testosterone levels were not affected by 1 to 100 nM OP; however, higher doses (500 and 2000 nM) reduced testosterone to 3.69 ± 0.17 and 2.61 ± 0.18 ng/10^5 cells, respectively ($P < 0.05$ when both compared to control). In response to 1 mM 8-Br-cAMP alone for 24 h, the testosterone level was 18.57 ± 0.63 ng/10^5 cells in immature Leydig cells (Fig. 2). Testosterone levels were unaffected by 1 or 10 nM OP; however, testosterone declined progressively to 16.38 ± 0.81, 14.81 ± 0.30, and 10.87 ± 0.27 ng/10^5 cells when exposed to 100, 500, and 2000 nM OP, respectively ($P < 0.05$ when each was compared to control). These studies suggest that the main actions of OP occur after the formation of cAMP in both precursor and immature Leydig cells.

3.3. Effect of 17β-estradiol on hCG-stimulated testosterone

Because OP has been reported to behave as a weak estrogen in several cellular systems [10,12], the response of cultured precursor and immature Leydig cells to increasing

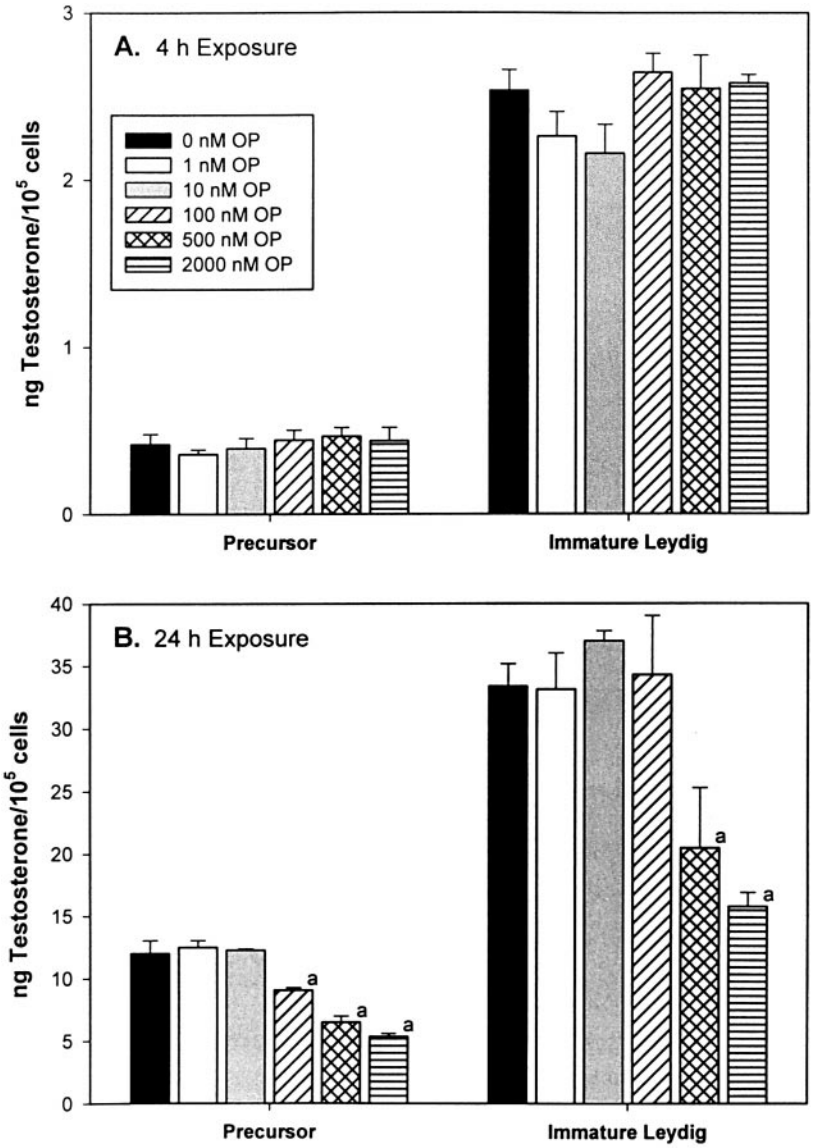

Fig. 1. Effect of octylphenol on hCG-stimulated testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. All cells received 1 μM 4-MOPC to inhibit 5α-reductase activity. Cells received varying concentrations of OP (1 to 2000 nM), dissolved in ethanol, before treatment with 10 mIU/mL hCG. The final ethanol concentration in all treatment groups, including control, was 0.1%. Cells were exposed for 4 (A) or 24 h (B). After treatment, media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate studies. *$P < 0.05$ when compared to appropriate control.

17β-estradiol and 10 mIU/mL hCG was evaluated. In response to hCG alone for 4 h, precursor cells produced 3.01 ± 0.26 ng testosterone/10^5 cells (Fig. 3A). Increasing 17β-estradiol concentrations (1 to 1000 nM) had no effect on hCG-stimulated testosterone. In response to hCG alone for 4 h, immature Leydig cells produced 5.68 ± 0.88 ng testosterone/10^5 cells (Fig. 3A). Exposure to 17β-estradiol (1 to 1000 nM) had no effect on testosterone formation. Extending the exposure time to 17β-estradiol (1 to 1000 nM) to 24 h similarly had no effect on hCG-stimulated testosterone formation in precursor or immature Leydig cells (Fig. 3B). Extending the exposure time to 17β-estradiol to 3 or 5 days also had no effect on hCG-stimulated testosterone in both cell types (data not shown). Furthermore, substituting DES as the estrogen agonist in these studies produced similar results (data not shown). These studies demonstrate that OP is not mimicking the actions of estrogen on precursor and immature Leydig cell testosterone biosynthesis.

3.4. Effect of ICI 182,780 on the effects of OP on hCG-stimulated testosterone

ICI 182,780 (ICI) is a pure estrogen antagonist that block estrogen effects mediated through either estrogen receptor α (ERα) or estrogen receptor β (ERβ) [38]. Cultured precursor

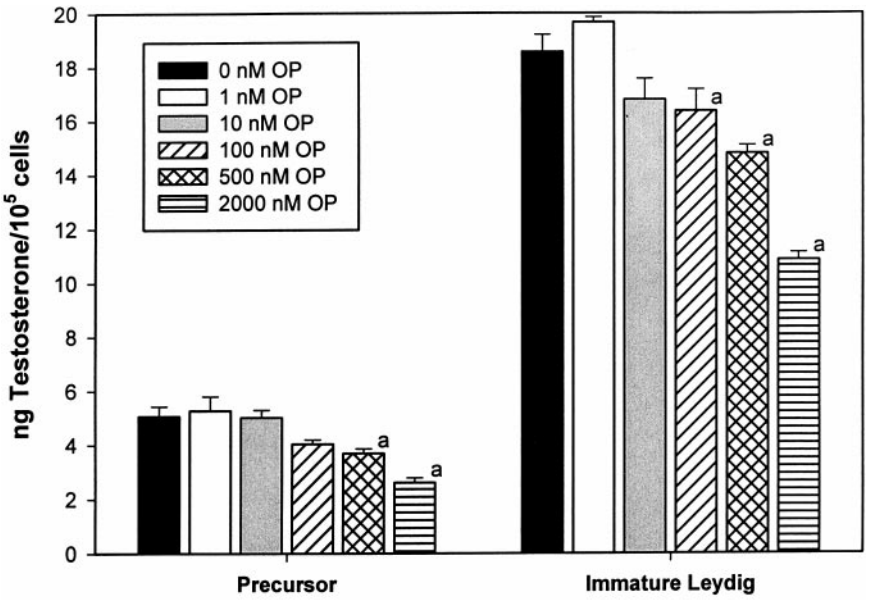

Fig. 2. Effect of octylphenol on 8-Br-cAMP-stimulated testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h before media change and initiation of treatment. All cells received 1 μM 4-MOPC to inhibit 5α-reductase activity. Cells received varying concentrations of OP (1 to 2000 nM), dissolved in ethanol, before treatment with 1 mM 8-Br-cAMP. The final ethanol concentration in all treatment groups was 0.1%. The total treatment time was 24 h after which media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate studies. *$P < 0.05$ when compared to appropriate control.

and immature Leydig cells were treated concomitantly with 100 nM ICI, increasing OP concentrations (1 to 2000 nM), and 10 mIU/mL hCG for 24 h to determine whether the actions of OP on testosterone formation were mediated through binding to ERα or ERβ. The testosterone level of precursor cells treated with ICI and hCG was 2.59 ± 0.23 ng/10^5 cells (Fig. 4). Significant declines in testosterone were not observed after exposure to 1 to 100 nM OP; however, higher OP concentrations (500 and 2000 nM) reduced testosterone to 1.79 ± 0.15 and 1.44 ± 0.16 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control). The testosterone level of immature Leydig cells exposed to ICI and hCG was 15.58 ± 0.78 ng/10^5 cells (Fig. 4). OP concentrations of 1 or 10 nM had no effect on testosterone levels; however, levels declined progressively to 11.89 ± 0.56, 11.06 ± 0.55, and 9.60 ± 0.57 ng/10^5 cells at 100, 500, and 2000 nM OP, respectively ($P < 0.05$ when each compared to control). Exposure to higher ICI concentrations (200 or 500 nM) similarly did not alter the effects of OP (data not shown). These studies demonstrate that the inclusion of ICI did not alter the pattern of inhibition on testosterone biosynthesis elicited by higher OP concentrations in precursor or immature Leydig cells, suggesting that the effects of OP are not mediated through binding to ERα or ERβ.

3.5. Effects of OP and hCG exposure on conversion of androgen precursors to testosterone

To further localize potential site(s) of action of OP, cultured precursor and immature Leydig cells were exposed to androgen precursors after incubation with increasing concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h. Fresh media containing 1 μM 4-MOPC and 1 μM each of 22(R)-hydroxycholesterol, pregnenolone, progesterone, or androstenedione were added to each well, and the treated cells were incubated for 4 h at 33°C. Media were collected, and testosterone levels were determined by RIA.

The amount of 22(R)-hydroxycholesterol converted to testosterone in control precursor cells was 0.75 ± 0.05 ng/10^5 cells (Fig. 5A). Both 1 and 10 nM OP-exposed cells produced similar testosterone levels. However, the conversion of 22(R)-hydroxycholesterol to testosterone declined progressively to 0.50 ± 0.03, 0.38 ± 0.02, and 0.27 ± 0.02 ng/10^5 cells in cells exposed to 100, 500, and 2000 nM OP, respectively ($P < 0.05$ when each was compared to control). The conversion of 22(R)-hydroxycholesterol to testosterone in control immature Leydig cells was 0.56 ± 0.04 ng/10^5 cells (Fig. 5A). Testosterone levels were not altered in cells exposed to 1, 10, or 100 nM OP. However, exposure to 500 or 2000 nM OP significantly reduced 22(R)-hydroxycholesterol conversion to testosterone to 0.28 ± 0.03 and 0.17 ± 0.02 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control).

The conversion of pregnenolone to testosterone in control precursor cells was 0.78 ± 0.08 ng/10^5 cells (Fig. 5B). Exposure to 1 or 10 nM OP had no effect on testosterone levels; however, exposure to 100, 500, or 2000 nM OP progressively reduced testosterone levels to 0.53 ± 0.04, 0.42 ± 0.01, and 0.30 ± 0.03 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control). Testoster-

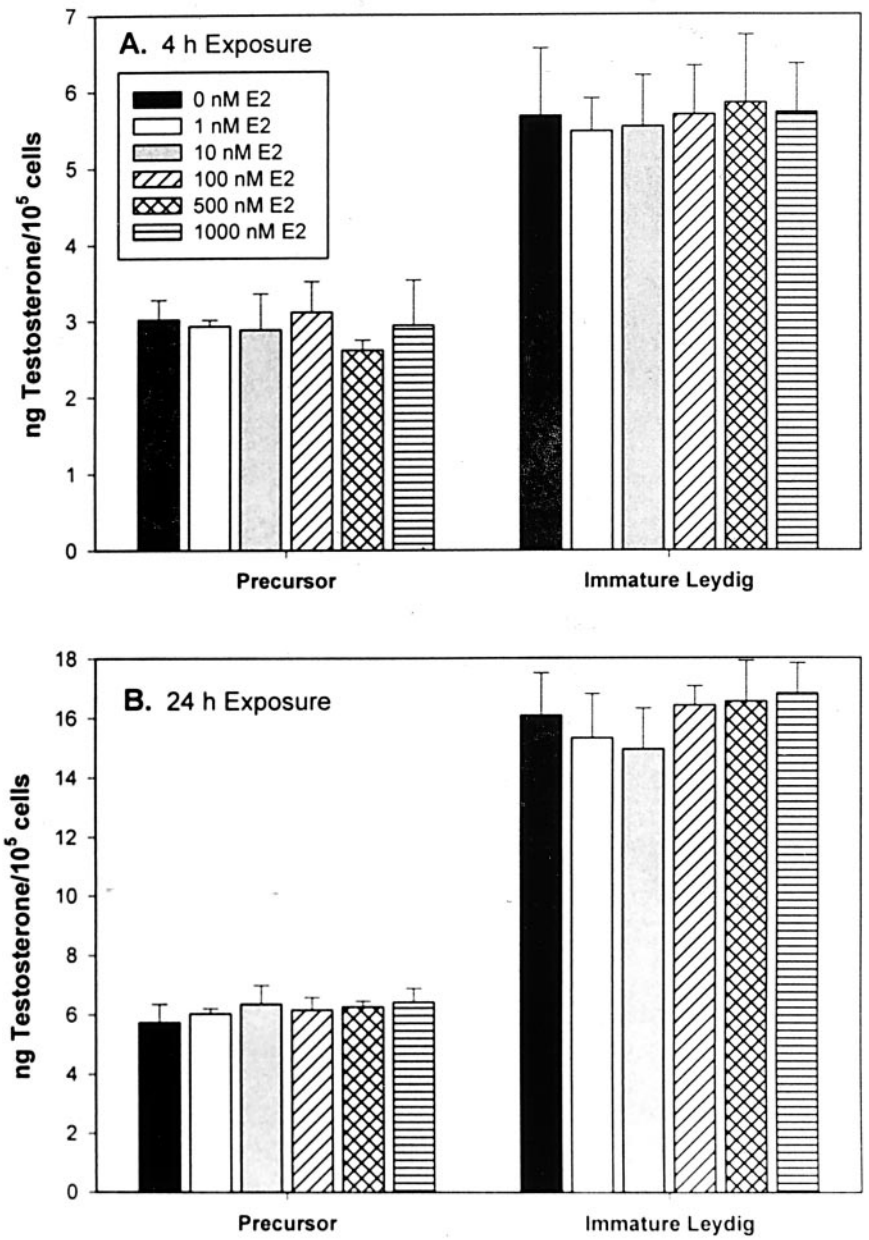

Fig. 3. Effect of 17β-estradiol on hCG-stimulated testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. All cells received 1 μM 4-MOPC to inhibit 5α-reductase activity. Cells received varying concentrations of 17β-estradiol (1 to 1000 nM), dissolved in ethanol, before treatment with 10 mIU/mL hCG. The final ethanol concentration in all treatment groups was 0.1%. Cells were treated for four (A) or 24 h (B) after which media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate studies.

one formation from pregnenolone as substrate in control immature Leydig cells was 1.36 ± 0.06 ng/10^5 cells (Fig. 5B). Testosterone levels were not affected by exposure to 1 or 10 nM OP; however, higher OP concentrations (100, 500, or 2000 nM) significantly reduced testosterone levels to 0.93 ± 0.03, 0.64 ± 0.02, and 0.44 ± 0.04 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control).

The conversion of progesterone to testosterone in control precursor cells was 0.90 ± 0.04 ng/10^5 cells (Fig. 5C). Exposure to lower OP concentrations (1 or 10 nM) had no effect on testosterone levels; however, exposure to higher OP concentrations (100, 500, or 2000 nM) significantly reduced testosterone levels to 0.59 ± 0.04, 0.49 ± 0.04, and 0.40 ± 0.02 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control). The conversion of progesterone to testosterone in control immature Leydig cells was 1.12 ± 0.03 ng/10^5 cells (Fig. 5C). Testosterone levels were unaffected by prior exposure to 1, 10, or 100 nM OP; however, exposure to 500 or 2000 nM OP significantly reduced testosterone levels to 0.58 ± 0.05 and 0.41 ± 0.04 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control).

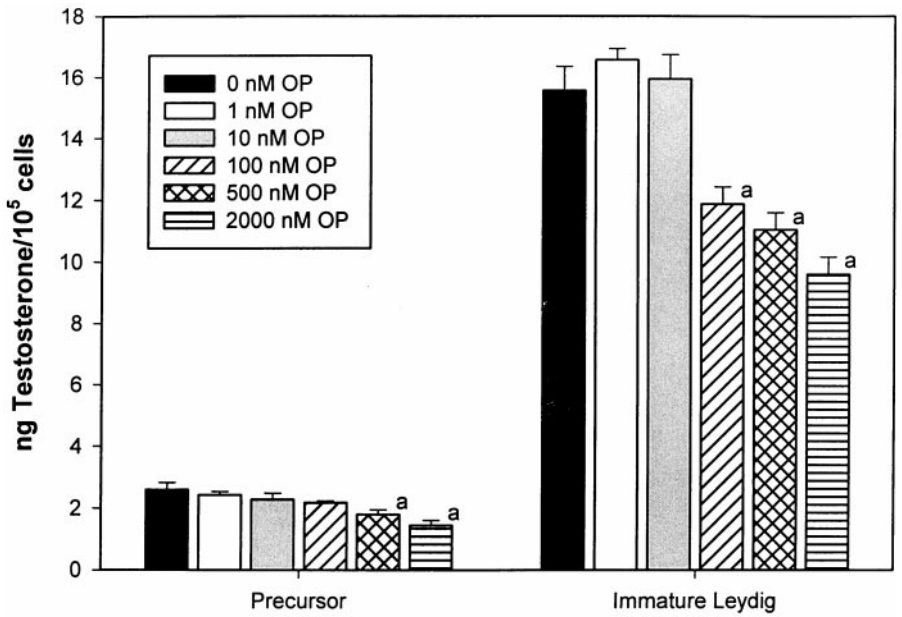

Fig. 4. Effect of ICI 182,780 on effects of octylphenol on hCG-stimulated testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. Cells received 1 μM 4-MOPC, 100 nM ICI, and varying concentrations of OP (1 to 2000 nM), dissolved in ethanol, before treatment with 10 mIU/mL hCG. The final ethanol concentration in all treatment groups was 0.1%. After treatment for 24 h, media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate experiments. *$P < 0.05$ when compared to appropriate control.

The conversion of androstenedione to testosterone in control precursor or immature Leydig cells was 9.32 ± 0.62 and 18.24 ± 1.97 ng/10^5 cells, respectively (Fig. 5D). The formation of testosterone from androstenedione was unaffected by exposure to any of the OP concentrations examined.

The conversion of cholesterol to testosterone in precursor or immature Leydig cells involves four enzymatic steps: 1) mitochondrial P450 side-chain cleavage activity (P450scc), which converts cholesterol to pregnenolone; 2) 3β-hydroxysteroid dehydrogenase-isomerase type I (3β-HSD), which converts pregnenolone to progesterone; 3) 17α- hydroxylase/c17–20-lyase (P450c17) which converts progesterone to androstenedione; and 4) 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD) which converts androstenedione directly to testosterone. These substrate conversion studies demonstrate that 17β-HSD activity is unaffected by OP in both precursor and immature Leydig cells and indicate that the P450c17 step is inhibited in both cell types. It remains to be established whether P450scc and/or 3β-HSD activities are affected by OP.

3.6. Effect of concomitant exposure to antioxidants on effects of OP on hCG-stimulated testosterone

In the presence of 20 μM α-tocopherol, 10 mIU/mL hCG-stimulated testosterone level was 2.98 ± 0.18 ng/10^5 precursor cells after 24 h of exposure (Fig. 6A). Lower OP concentrations (1 and 10 nM) had no effect on testosterone levels in precursor cells; however, higher concentrations (100, 500, and 2000 nM) progressively decreased testosterone to 1.54 ± 0.03 ng/10^5 cells at the highest concentration ($P < 0.05$ when each was compared to control). In the presence of 20 μM α-tocopherol, the 10 mIU/mL hCG-stimulated testosterone level was 12.67 ± 0.29 ng/10^5 immature Leydig cells (Fig. 6A). Testosterone levels were not altered in cells exposed to 1, 10, and 100 nM OP; however, higher OP concentrations (500 and 2000 nM) progressively reduced testosterone to 8.24 ± 0.49 ng/10^5 cells at the highest OP concentration ($P < 0.05$ when each was compared to control). The evaluation of higher α-tocopherol concentrations (100 and 200 μM), similarly, did not alter the inhibiting effects of higher OP concentrations (data not shown).

In the presence of 100 μM ascorbate, the 10 mIU/mL hCG-stimulated testosterone level was 2.96 ± 0.02 ng/10^5 precursor cells after 24 h of exposure (Fig. 6B). Concentrations of OP from 1 to 100 nM had no effect on testosterone; however, higher OP concentrations (500 and 2000 nM) progressively decreased testosterone to 1.99 ± 0.07 and 1.61 ± 0.06 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control). In the presence of 100 μM ascorbate, the 10 mIU/ml hCG-stimulated testosterone level was 11.69 ± 0.06 ng/10^5 immature Leydig cells after 24 h of exposure. Concentrations of OP from 1 to 100 nM had no effect on testosterone levels; however, 500 and 2000 nM OP progressively decreased testosterone to 9.29 ± 0.49 and 8.25 ± 0.46 ng/10^5 cells, respectively ($P < 0.05$ when each was compared to control). The inclusion of higher ascorbate levels (200 and 500 μM), similarly, did not alter the inhibiting effects of higher OP concentrations (data not shown).

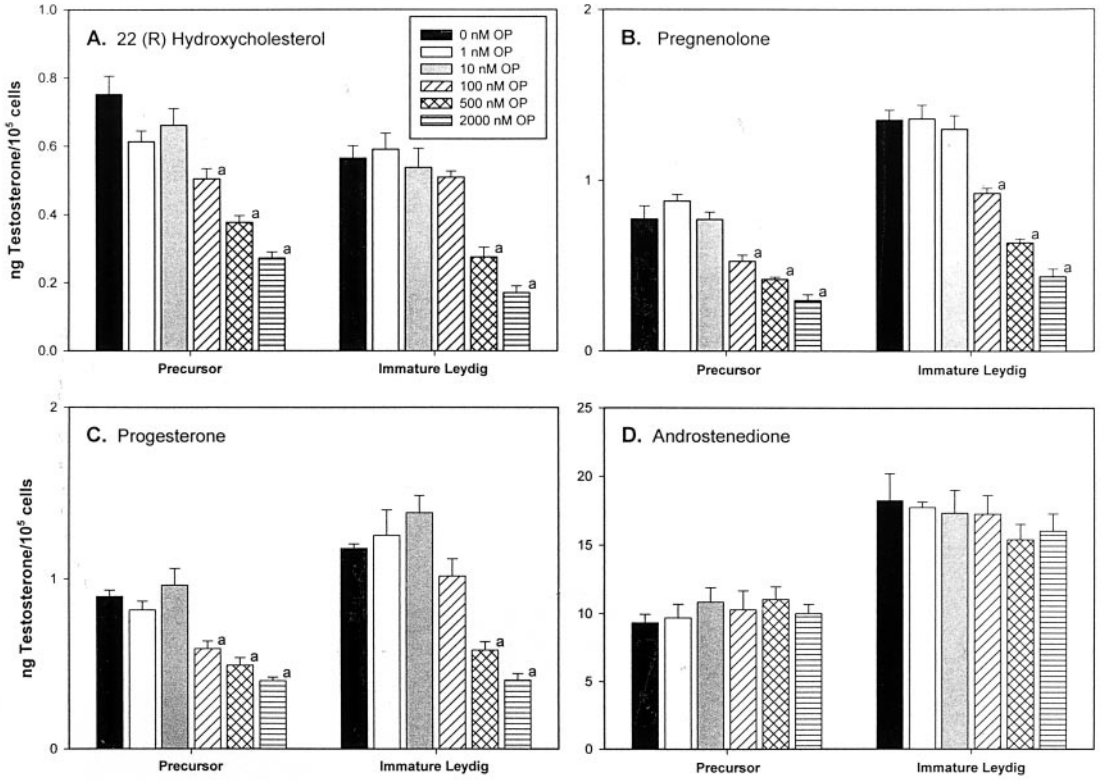

Fig. 5. Effect of exposure to octylphenol and hCG on steroid substrate conversion to testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. Cells were exposed to 1 μM 4-MOPC, varying concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for 24 h. Fresh media containing 1 μM 4-MOPC were added to all wells, and cells were exposed to 1 μM 22(R)-hydroxycholesterol (A), 1 μM pregnenolone (B), 1 μM progesterone (C), or 1 μM androstenedione (D). The final ethanol concentration in each well was 0.1%. Cells were incubated for 4 h at 33°C. Media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate experiments. *$P < 0.05$ when compared to appropriate control.

Thus, the antioxidants α-tocopherol and ascorbate did not alter the inhibitive effects of higher OP concentrations on hCG-stimulated testosterone formation by cultured precursor and immature Leydig cells.

3.7. Effect of OP on ^{125}I-hCG binding

The specific binding of ^{125}I-hCG to precursor or immature Leydig cells indirectly measures LH receptor (LHR) numbers on these cells [34]. Whether exposure of precursor or immature Leydig cells to increasing concentrations of OP (1 to 2000 nM) for 24 h alters the number of LHR was evaluated. Because exposure to hCG causes down regulation of LHR in both immature and adult Leydig cells [39], the effect of OP on ^{125}I-hCG binding to LHR in precursor and immature Leydig cells was evaluated under basal conditions. ^{125}I-hCG binding to LHR in precursor cells not exposed to OP was 962 ± 17 cpm/well (Fig. 7). Increasing concentrations of OP (1 to 2000 nM) did not significantly alter binding to LHR in precursor cells. In the absence of OP, ^{125}I-hCG binding to LHR in immature Leydig cells was 1404 ± 30 cpm/well, and increasing OP concentrations (1 to 2000 nM) had no effect on the level of binding (Fig. 7).

3.8. Effect of octylphenol on hCG-stimulated cAMP levels

The basal cellular cAMP level of cultured precursor cells after 4 h of culture was 48.4 ± 1.1 fmol/well. Treatment with 10 mIU/mL hCG for 4 h and the inclusion of 0.1 mM IBMX increased the cellular cAMP level to 1235.2 ± 199.9 fmol/well, and this level was unaffected by exposure to increasing concentrations of OP (1 to 2000 nM) (Fig. 8A). The basal cellular cAMP level of cultured immature Leydig cells after 4 h of culture was 26.1 ± 0.1 fmol/well. Treatment with hCG for 4 h increased the cAMP level to 3360.5 ± 297.1 fmol/well, which was not affected by the inclusion of increasing concentrations of OP (Fig. 8A).

The basal cellular cAMP level of cultured precursor cells after 24 h of culture was 36.7 ± 0.7 fmol/well. Treatment with hCG for 24 h elevated the cAMP levels to 134.4 ± 4.1 fmol/well (Fig. 8B), which represented a decline from the 4 h level. Exposure to increasing concentrations of OP (1 to 2000 nM) had no effect on the level of cAMP. The basal cellular cAMP level of cultured immature Leydig cells after 24 h of culture was 18.5 ± 1.5 fmol/well. Treatment with hCG increased cAMP to

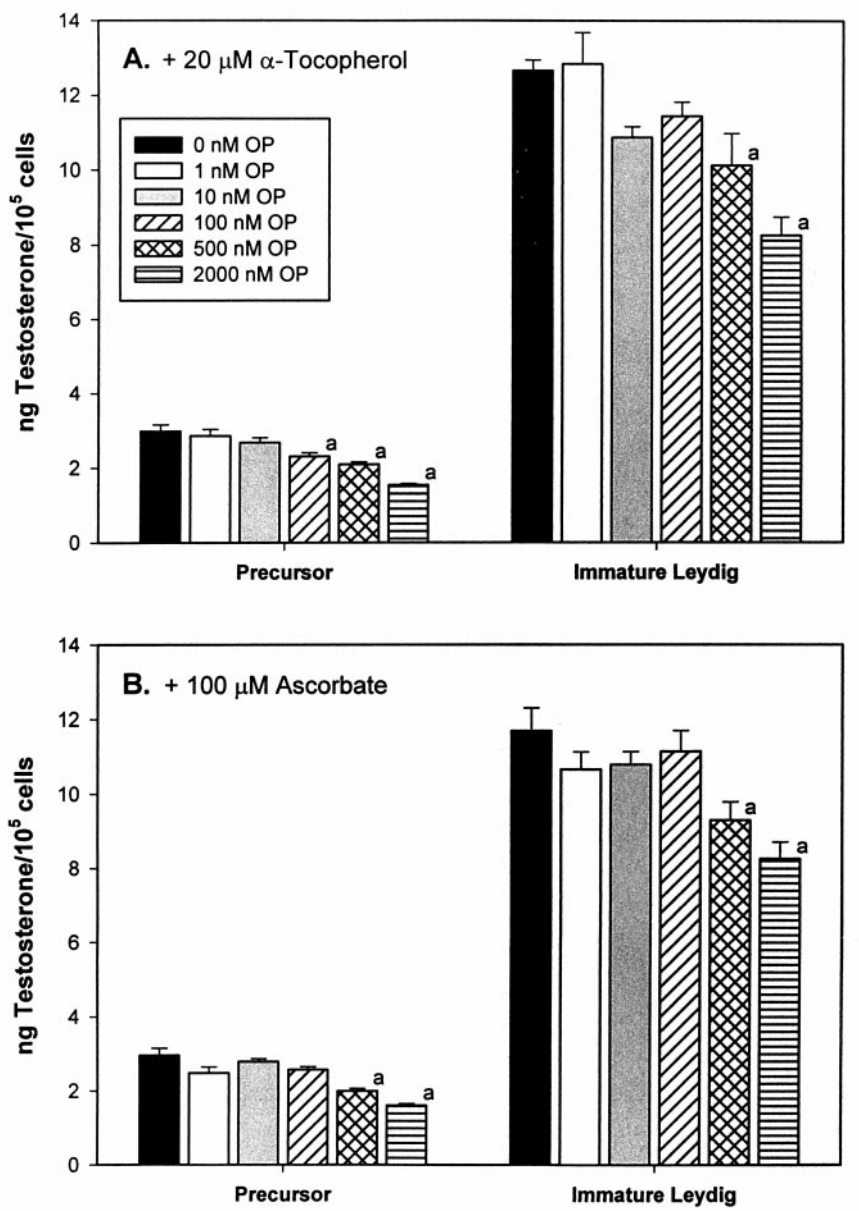

Fig. 6. Effect of exposure to antioxidants on effects of octylphenol on hCG-stimulated testosterone in precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. All cells received 1 μM 4-MOPC and either 20 μM α-tocopherol (A) or 100 μM ascorbate (B). Then cells were exposed to varying concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG. The final ethanol concentration in all treatment groups was 0.1%. The exposure period was 24 h, after which media were collected for quantitation of testosterone by RIA. Each value represents the mean ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate studies. *$P < 0.05$ when compared to control.

239.2 ± 13.8 fmol/well (Fig. 8B), which represented a decline from the 4-h level. Exposure to increasing concentrations of OP had no effect on the 24 h hCG-stimulated cAMP level. These results suggest that the primary action of OP on hCG-stimulated testosterone formation in both cultured precursor and immature Leydig cells occurs after the formation of cAMP. In addition, because cellular cAMP levels were unaffected by OP, these results are an indirect measure of cellular viability.

4. Discussion

The current studies demonstrate that direct exposure to increasing OP concentrations (1 to 2000 nM) inhibits hCG-stimulated testosterone biosynthesis by both cultured rat precursor and immature Leydig cells. Significant declines in testosterone biosynthesis were observed starting at 100 or 500 nM OP, with further progressive declines at higher concentrations. Maximal declines in testosterone at the highest OP concentration evaluated in both precursor and

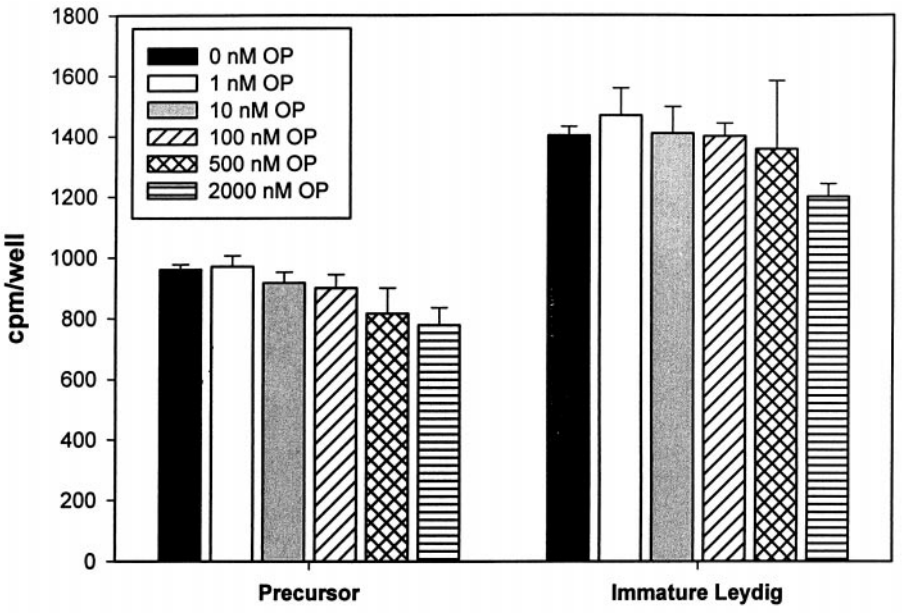

Fig. 7. Effect of octylphenol on ^{125}I-hCG binding to precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. All cells received 1 μM 4-MOPC and varying concentrations of OP (1 to 2000 nM) for 24 h. Next, fresh media containing a saturating concentration of ^{125}I-hCG was added, and cells were incubated for 3 h at 33°C. Separate samples 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 ± SEM of four separate samples from a single experiment, and these results are representative of at least three separate studies.

immature Leydig cells ranged from ~ 30 to 70% below control. The primary effects of OP appeared to be localized between enzymatic steps converting cholesterol to androstenedione. 17β-HSD activity, which converts androstenedione to testosterone, was not affected. P450c17 activity, which converts progesterone to androstenedione, was inhibited by OP. It remains to be established whether P450scc activity, which converts cholesterol to pregnenolone, and/or 3β-HSD activity, which converts pregnenolone to progesterone also are inhibited by OP.

A previous study reported that increasing concentrations of OP had a biphasic effect on hCG-stimulated testosterone biosynthesis in cultured neonatal rat Leydig cells, with lower concentrations (1 and 10 nM) actually increasing testosterone biosynthesis, whereas higher concentrations (100 to 2000 nM) progressively decreased testosterone from peak levels [20]. Furthermore, these effects appeared to be localized after the formation of cAMP and before the P450scc step [20]. In the current studies using both rat precursor and immature Leydig cells, only inhibiting effects of higher OP concentrations were observed, and this effect seemed to be localized before the formation of androstenedione. Although the pattern of inhibition to OP in both precursor and immature Leydig cells seemed to be similar, their pattern of response and site(s) affected differed from that of cultured neonatal Leydig cells. This finding is not surprising because precursor and immature rat Leydig cells represent a different population from Leydig cells found in neonatal animals. Leydig cells present in neonatal rats represent fetal Leydig cells that persist for about 2 weeks after birth, then lose their functional capacity [23]; however, precursor Leydig cells are mesenchymal cells that differentiate into immature Leydig cells between the second and fourth weeks after birth [24], mainly through the actions of LH [25] and possibly androgen [34]. In addition, neonatal (fetal) rat Leydig cells differ from precursor and immature Leydig cells in several functional aspects. They exhibit very low 5α-reductase activity so that testosterone is the main androgen secreted [40], and they are not desensitized after exposure to a dose of hCG as is the case for immature or adult-type Leydig cells [41].

We reported previously that 17β-estradiol (1 to 1000 nM) had no effect on hCG-stimulated testosterone biosynthesis by cultured neonatal rat Leydig cells and that the biphasic pattern of response to OP was not affected by concomitant exposure of cells to the pure estrogen antagonist, ICI [20]. In the present studies, 17β-estradiol (1 to 1000 nM) similarly had no effect on hCG-stimulated testosterone formation in both precursor and immature Leydig cells, and ICI did not alter the inhibiting effects of higher OP concentrations in either cell type. Although both ERα and ERβ have been localized in rat Leydig cells during different stages of maturation [42,43], and OP has been reported to behave as a weak estrogen in several cellular systems [11,12], the actions of OP on hCG-stimulated testosterone biosynthesis in cultured precursor and immature Leydig cells in the present study and in neonatal Leydig cells [20], do not appear to be mediated through the classic ERα or ERβ. With respect to the direct actions of 17β-estradiol on LH/hCG-stimulated testosterone biosynthesis by rat Leydig cells, two previous studies reported that only high concentrations (above 180 μM) inhibited testosterone

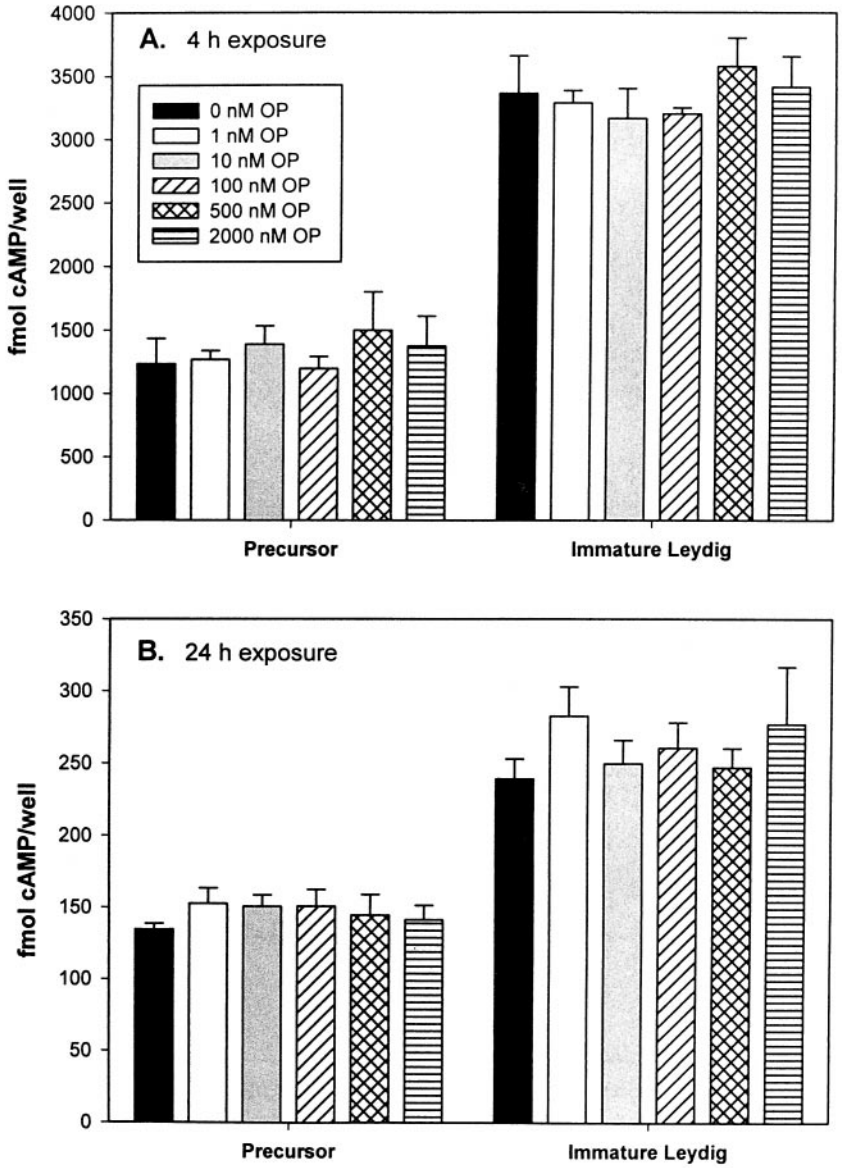

Fig. 8. Effect of octylphenol on cellular cAMP levels of cultured precursor and immature Leydig cells. Precursor and immature Leydig cells were cultured for ~ 20 h after plating before media change and initiation of treatment. Cells were exposed to 0.1 mM IBMX, varying concentrations of OP (1 to 2000 nM) and 10 mIU/mL hCG for four (A) or 24 h (B). After treatment cellular cAMP was extracted, and cAMP levels were quantitated by RIA as described previously in Section 2 (measurement of cellular cAMP).

biosynthesis [44,45]. In contrast, in vivo exposure of adult hypophysectomized (hypox) rats to 17β-estradiol decreased plasma testosterone levels [46] and the sensitivity of isolated Leydig cells in producing testosterone in response to LH [47]. Furthermore, there was evidence that in vivo exposure of hypox rats to 17β-estradiol reduced P450c17 activity [48]. We are not aware of a valid explanation of why in vitro Leydig cell responses to 17β-estradiol differ from in vivo responses, although potential indirect actions of 17β-estradiol in vivo (CNS involvement or paracrine actions within the testis) may play a role. Nevertheless, because OP directly inhibits hCG-stimulated testosterone biosynthesis by both precursor and immature Leydig cells whereas 17β-estradiol does not, it is apparent that some OP effects must be separate from that of the native estrogen.

Although the results of the present study suggest that OP directly inhibits P450c17 activity and potentially P450scc and/or 3β-HSD activities in precursor and immature Leydig cells, these results do not define how OP mediates this effect(s). Because the effects of OP on hCG-stimulated testosterone biosynthesis do not appear to be mediated through the ER, the possibility that OP actions could be explained by its functioning as a pro-oxidant was evaluated. The conversion of cholesterol to testosterone by Leydig cells involves the participation of two cytochrome P450 enzymes (P450scc and P450c17). These enzymes use mo-

lecular oxygen and electrons provided from NADPH for hydroxylation of the substrate. During normal steroidogenesis, reactive oxygen species (superoxide and/or hydroxy radicals) are produced by electron leakage outside the electron transfer chains [49,50], and these radicals can initiate lipid peroxidation to inactivate P450 enzymes [51]. Antioxidants such as α-tocopherol or ascorbate can protect P450 enzymes from lipid peroxidative damage [52]. Normal products of steroid biosynthesis 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 [52]. This phenomenon can lead to electron leakage and free radical formation [52] Thus, compounds that act as pseudosubstrates could inhibit steroid production. Because the decline in hCG-stimulated testosterone formation by higher OP concentrations was not altered by concomitant exposure to α-tocopherol or ascorbate, it does not appear that OP is functioning as a pseudosubstrate in the generation of free radicals and the inhibition of testosterone formation in precursor or immature Leydig cells.

The current in vitro results must be evaluated in light of recent multigeneration in vivo data in rats [21]. In this study, the administration of nonylphenol (NP) in the diet of Sprague–Dawley rats for three generations produced minimal effects on the male reproductive system of exposed animals. The limited effects of NP in vivo may suggest: 1) that there are inherent differences between in vitro and in vivo responses to alkylphenols, 2) that any declines in testosterone levels in vivo may not have been adequate to affect spermatogenesis or target organ responses to testosterone and/or 3) that responses of the male reproductive system to NP may differ from its sensitivity to OP. The later explanation is possible as OP was much more potent than NP in stimulating vitellogenin production by cultured trout hepatocytes and in stimulating transcriptional activity and growth of cultured MCF-7 human breast cancer cells [10]. Whatever the reason, it will be important to ascertain why these differences exist.

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