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Exposure to octylphenol increases basal testosterone formation by cultured adult rat Leydig cells

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Abstract

4-*Tert*-octylphenol (OP) is a breakdown product of 4-*tert*-octylphenol ethoxylate, which is a surfactant additive widely used in the manufacture of a variety of detergents and plastic products. OP has been reported to exhibit weak estrogenic activity in many assay systems. The studies described herein examined an unusual effect of OP in increasing constitutive testosterone levels of cultured Leydig cells from young adult rats. The increase in testosterone was both dose and time sensitive, and this response was observed in medium lacking both calcium and magnesium and containing a membrane-permeable calcium chelator, suggesting that the increase in testosterone was not mediated by an increase in the permeability of extracellular calcium into cells or the redistribution/release of calcium from intracellular stores, respectively. Cellular cAMP levels also were unaffected by OP alone in cultured Leydig cells. Furthermore, initial exposure to 2000 nM OP alone for 4 h did not alter the subsequent conversion of endogenous cholesterol or exogenously added 22 (R)hydroxycholesterol to testosterone, suggesting that the increase in testosterone was not due to the enhanced availability of endogenous cholesterol or an increase in cholesterol side-chain cleavage activity, respectively. The increase in testosterone also was observed in the presence of the pure estrogen antagonist, ICI 182,780, or a 5α-reductase inhibitor, suggesting that this effect of OP was not mediated through the estrogen receptor α or β pathway or by inhibition of Leydig cell testosterone metabolism, respectively. In addition, exposure of cells to comparable concentrations of two different detergents, Triton X-100 or sodium cholate, did not increase testosterone levels, suggesting that this effect of OP was not due to its potential detergent qualities. Although these studies did not identify specific mechanism(s) that increase constitutive testosterone levels by OP, they identify specific pathways that appear not to be involved. The physiological relevance of this observation is not known; nevertheless, they illustrate potential diverse actions of OP in modulating the level of androgen secreted by Leydig cells, and they emphasize that some actions of OP do not appear to be mediated through the estrogen receptor α or β pathway.

Keywords: Octylphenol; Leydig cell; Testosterone

1. Introduction

It has been hypothesized that environmental/occupational exposure to endocrine-disrupting chemicals has adversely affected the reproductive system of humans and various wildlife species [1,2]. Recent changes in males that have occurred over the past 40–50 years ascribed to gestational exposure to these chemicals [3] include: (1) an international decline in semen quality [4]; (2) a rise in the incidence of cryptorchidism [5]; and (3) an increase in the incidence of testicular cancer [6,7]. However, this proposal is not universally accepted [8], and it has not been established whether such changes have adversely affected male fertility [9]. Although there is disagreement over the extent to which exposure to endocrine-disrupting chemicals has adversely affected male reproductive functions, there is general agreement that additional studies are needed to clarify the association between exposure and potential effects on human reproductive health [10].

Alkylphenol ethoxylates (APEs) are a class of nonionic surfactants widely used in the manufacture of plastics, detergents, paints and pesticides [11]. The alkyl groups mainly are branched nonyl or octyl chains positioned opposite the *para*-substituted ethoxylate chain, composed of 1–100 repeating ethylene oxide units. 4-*Tert*-octylphenol (OP) and 4-nonylphenol (NP) are environmentally persistent degradation products of APEs [12]. Both OP and NP have been reported to exhibit weak estrogenic activity in various testing assays. They were demonstrated to stimulate growth [13] and to displace ^{3}H-estradiol binding to the estrogen receptor [14] of MCF-7 human breast cancer cells, to stimulate

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estrogen-dependent β-galactosidase activity in a yeast estrogen screen assay [15] and to stimulate uterine growth following in vivo administration to immature rats [16].

The majority of APEs used in the US enters the aquatic environment following usage, and the highest concentrations of alkylphenols (APs) appear to be found in water ways receiving industrial or municipal waste water [12] or in sewage sludge [17]. Although the levels of APs in river water downstream of these sites were in the low nanomolar range, river sediment contained 0.02–15 μM range of APs [12], and sewage sludge contained millimolar levels of APs [17]. Thus, although potential exposure levels to APs and, APEs are generally low, under special conditions, exposure to higher levels is possible.

It has been reported that gestational exposure to estrogenic chemicals can adversely affect reproductive functions of both male and female offspring. Male offspring of mothers treated with diethylstilbestrol (DES), a synthetic estrogen used in the 1950s and 1960s to prevent complications of pregnancy, were reported to have a higher incidence of testicular hypoplasia, semen abnormalities and cryptorchidism [18]. Similar effects on male offspring of pregnant mice exposed to DES have been reported [19,20], and gestational exposure of pregnant rats to DES or OP was reported to decrease the amount of the steroidogenic enzyme 17α-hydroxylase/c17–20 lyase (P450c17), and of steroidogenic factor 1 (SF-1), a transcription factor involved in the development of adrenals and gonads and the expression of steroidogenic enzymes [21,22]. In addition, exposure of neonatal rats to NP reduced the size of the testis and accessory sexual organ weights and increased the incidence of cryptorchidism [23]. In adult rats, chronic exposure to OP or estradiol valerate reduced testis size and sperm numbers [24], and in cultured Leydig cells, OP inhibited human chorionic gonadotropin (hCG)-stimulated testosterone formation [25]. In cultured mouse Leydig tumor cells, OP inhibited hCG-stimulated cAMP formation and both basal and hCG-stimulated progesterone biosynthesis [26]. Thus, in general, the effects of APs on testicular function are inhibitive, and their effects are attributed to their estrogen-mimicking activity. However, in a recent study, it was reported that exposure of cultured Leydig cells from young adult rats to OP alone for 4 h increased testosterone levels approximately two-fold above control levels [25]. The current studies evaluated potential mechanisms by which this enhanced formation of androgen was mediated.

2. Materials and methods

2.1. Animals

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

2.2. Reagents

Collagenase (Type I), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNase I), Triton X-100, 22(R)-hydroxycholesterol, sodium cholate, 1, 2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′*N*′ -tetraacetic acid tetrakis (acetoxy-methyl ester) (BAPTA) and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma, St. Louis, MO, USA. Bovine serum albumin (BSA, clinical reagent grade) and Ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA. Dulbecco's modified eagle medium (DMEM) without phenol red, F-12 nutrient mixture (F12) without phenol red, medium 199 (Med 199) Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++}, phosphate buffered saline (PBS, pH 7.4), 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) was from NEN Life Science Products, Boston, MA, USA. Testosterone was from Steraloids, Wilton, NH, USA. Sodium 4-aza-4-methyl-3-oxo-5-pregnan-20(s) carboxylate (4-MOPC), a 5α-reductase inhibitor effective on both types 1 and 2 isozymes, was a gift from Merck Research Laboratories, Rahway, NJ, USA. ICI 182,780 (ICI, a pure estrogen antagonist) was a gift from Dr. A.E. Wakeling (Zeneca Pharmaceuticals, Cheshire, England). 4-*Tert*-octylphenol (OP) was from Aldrich, Milwaukee, WI, USA. Percoll was from Pharmacia, Piscataway, NJ, USA.

2.3. Isolation and culture of Leydig cells

Animals were 57–65 days of age when sacrificed by intraperitoneal injection of pentobarbitol. To obtain sufficient cells for culture, testes from 8 to 10 animals were pooled. Testes were decapsulated and digested in 0.25 mg/ml collagenase in Med 199 + 0.1 BSA and 10 μg/ml DNase I for 20–30 min at 37 °C. The dispersed interstitial cells were elutriated using a Beckman elutriation apparatus as described previously [27]. Cells retained in the elutriation chamber were layered over a 60% Percoll gradient and centrifuged at ∼25,000 × *g* for 1 h. Leydig cells localized between densities of 1.07 and 1.09 g/ml.

Leydig cells were resuspended in a 1:1 mixture of DMEM/F12 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 [25]. One milliliter of cells (1×10^5 ml) was added into each 1.6 cm diameter well of a 24-well Costar culture plate and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 33 °C. Fresh medium without BSA was added ~20 h after plating, and treatments were initiated.

2.4. Treatment of cells

OP, ICI, Triton X-100, sodium cholate or 22(R)hydroxycholesterol were dissolved in ethanol. The final ethanol concentration in all treatment groups (including controls) was 0.1%. The concentrations of OP used in these experiments did not affect cell viability based on cell morphology, attachment to culture plate or the exclusion of trypan blue.

2.5. Quantitation of testosterone formation and cellular cAMP levels

Testosterone was quantitated directly from the culture medium by radioimmunoassay (RIA) as described previously [28]. Cellular cAMP content of cultured Leydig cells was quantitated after acetylation by RIA (Amersham cAMP Kit, no. RPA 509) as described previously [29].

2.6. Statistical analysis

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

3. Results

3.1. Effect of time of exposure to octylphenol on testosterone formation

Control and 2000 nM OP-exposed cells were cultured for 2, 4 and 8 h. Control cells produced 2.3 ng ± 0.2 ng, 5.0 ng ± 0.4 ng and 9.3 ng ± 0.4 ng testosterone/10^5 cells, respectively (Fig. 1). In response to 2000 nM OP, testosterone levels increased significantly to 5.6 ng ± 0.5 ng, 9.0 ng ± 0.8 ng and 21.3 ng ± 1.7 ng/10^5 cells, when compared to their respective controls. Exposure to shorter periods (0.5 and 1 h) to 2000 nM OP were examined; however, the modest increase at these times was not statistically significant (data not shown).

3.2. Effect of octylphenol on cellular cAMP levels

Leydig cells were exposed to increasing concentrations of OP (1–2000 nM) in the absence or presence of 100 μM IBMX for 4 h. OP did not significantly increase cellular cAMP levels under either condition (data not shown). Exposure to 2000 nM OP for shorter (0.5, 1, and 2 h) or longer (24 h) time periods also were evaluated; however, significant increases in cAMP levels above respective controls were not detected (data not shown).

3.3. Effect of Ca^{++} in mediating the effects of octylphenol on testosterone formation

Exposure of Leydig cells incubated in DMEM/F12 with increasing concentrations of OP (10–2000 nM) for 4 h increased testosterone from 2.8 ng ± 0.1 ng/10^5 cells

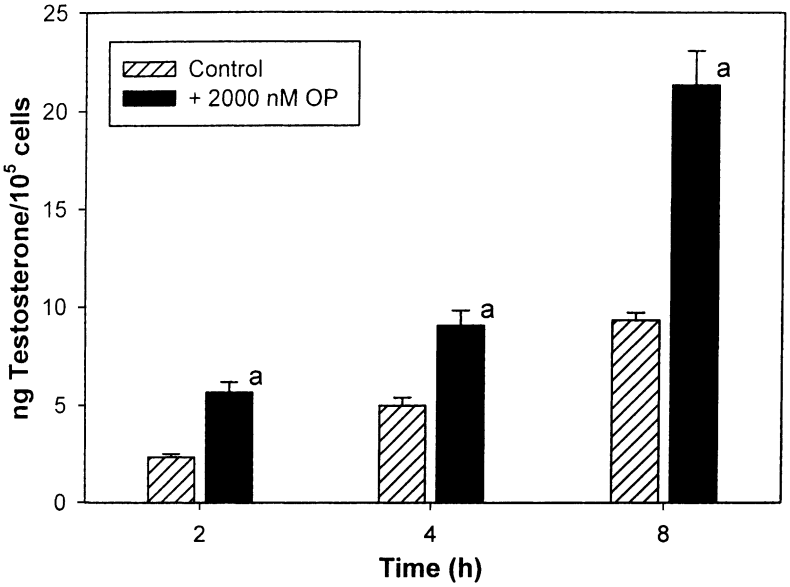

Fig. 1. Effect of time of exposure to octylphenol on testosterone formation by cultured Leydig cells. Leydig cells were exposed to 2000 nM OP or 0.1% ethanol (control) for 2, 4 or 8 h. Each value represents the mean ± S.E.M. of four samples from a single experiment. These results are representative of at least three separate experiments. a, $P < 0.01$ when compared with appropriate control.

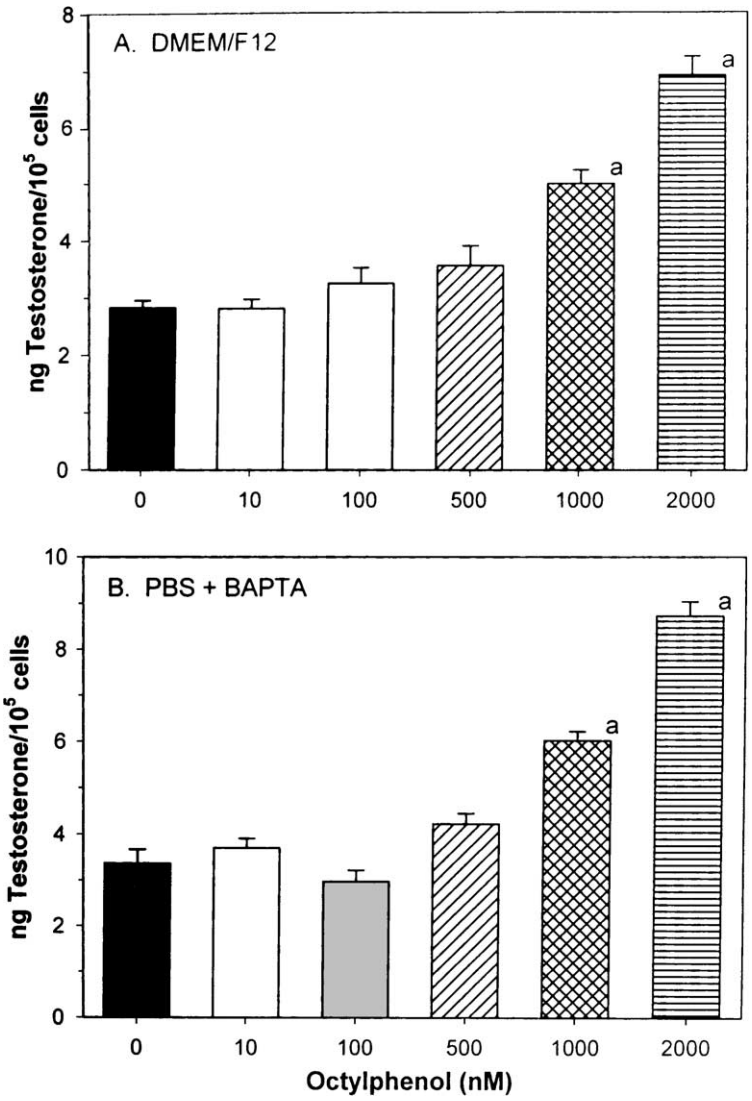

Fig. 2. Role of Ca^{++} on effect of octylphenol on testosterone formation of cultured Leydig cells. On day one of culture, fresh media containing DMEM/F12 (panel A) or PBS + 10 μM BAPTA (panel B) were added. After 15 min, increasing concentrations of OP were added, and testosterone levels were measured after 4 h. Each value represents the mean ± S.E.M. of four samples from a single experiment. These results are representative of at least three separate experiments. a, $P < 0.01$ when compared to appropriate control.

(control) to 5.0 ng ± 0.2 ng and 6.9 ng ± 0.3 ng/10^5 cells at 1000 and 2000 nM OP, respectively (Fig. 2, panel A). When the incubation medium was changed to PBS (to eliminate extracellular Ca^{++}) that included 10 μM BAPTA (a membrane-permeable Ca^{++} chelator, to tie up intracellular Ca^{++}), exposure of Leydig cells to increasing concentrations of OP (10–2000 nM) for 4 h increased testosterone levels at 1000 and 2000 nM OP to 1.8 and 2.6-fold greater than control, respectively (Fig. 2, panel B).

3.4. Effect of ICI 182,780 on the effect of octylphenol on testosterone formation

ICI is considered to be a "pure" estrogen antagonist [30] which is capable of blocking the transcriptional functions of estrogen mediated through estrogen receptor α or β [31]. Because adult rat Leydig cells express estrogen receptor α [32] and OP has been demonstrated to exhibit weak estrogenic activity [13–16], we evaluated whether the stimulatory effect of OP is mediated through the estrogen receptor. Leydig cells were exposed concomitantly to ICI (1 μM) and increasing concentrations of OP (10–2000 nM) for 4 h. Blocking estrogen receptor-mediated actions with ICI did not alter the increase in testosterone produced by higher OP concentrations (Fig. 3).

3.5. Role of cholesterol and/or cholesterol side-chain cleavage activity in mediating the effects of octylphenol on testosterone formation

To evaluate whether exposure to OP alone increases the provision of cholesterol to cholesterol side-chain cleavage

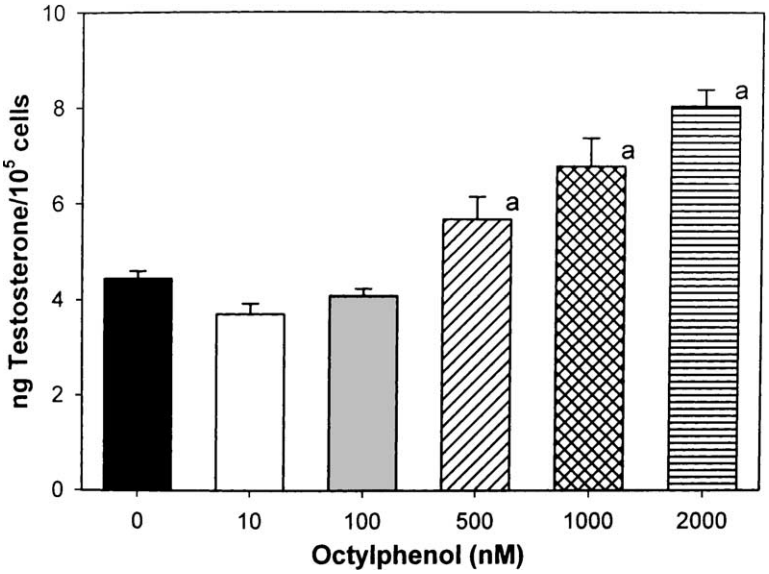

Fig. 3. Inclusion of ICI 182,780 on effect of octylphenol on testosterone formation of cultured Leydig cells. On day 1 of culture, fresh media (DMEM/F12) containing 1 μM ICI 182,780 were added. After 15 min, increasing concentrations of OP were added, and testosterone levels were measured after 4 h. Each value represents the mean ± S.E.M. of four samples from a single experiment. These results are representative of at least three separate experiments. a, $P < 0.05$ when compared with control.

activity (P450scc) and/or P450scc activity, itself, Leydig cells were exposed to 0.1% ethanol alone (control) or 2000 nM OP for 4 h. Next, fresh medium alone or fresh medium containing 10 μM 22(R)hydroxycholesterol was added to each well. Following incubation for 4 h, testosterone released into the medium was quantitated. Testosterone formation of OP-exposed cells was no different than control in cells incubated in media alone, suggesting that prior exposure to OP does not enhance the provision of endogenous cholesterol to mitochondrial P450scc (data not shown). Similarly, testosterone formation of OP-exposed cells incubated in media containing 10 μM 22(R)hydroxycholesterol was no different than control, suggesting exposure to OP alone does not increase P450scc activity(data not shown).

3.6. Effect of 4-MOPC on the effect of octylphenol on testosterone formation

Previous studies have demonstrated that immature rat testes express high 5α-reductase activity [33], which is localized mainly in Leydig cells [34]. After peaking in activity between days 30–40 after birth, 5α-reductase activity declines to reach low levels by about day 60 [33]. During this period when 5α-reductase activity is elevated, immature Leydig cells actively synthesize testosterone, but this androgen does not accumulate. Rather, it is converted to dihydrotestosterone (DHT), which, subsequently, is metabolized to 5α-androstane-3α,7β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol) [35]. We have reported previously that inhibition of 5α-reductase activity in immature rat Leydig cells leads to an increase in testosterone levels of treated cells [36]. With Leydig cell maturation (after about day 40 following birth), 5α-reductase activity progressively declines, and testosterone becomes the primary androgen. Although Leydig cell 5α-reductase activity of young adult rats (average ∼60 days of age in the current studies) would be expected to be very low and, consequently, not have an appreciable effect on testosterone accumulation, if residual activity still were present, possible inhibition of this enzyme by OP could increase testosterone levels of exposed cells.

To evaluate whether the increase in testosterone by exposure to OP was due to an inhibition of 5α-reductase activity, Leydig cells were exposed concomitantly to 1 μM 4-MOPC, a 5α-reductase inhibitor, and increasing concentrations of OP (10–2000 nM) for 4 h. Even in the presence of 4-MOPC, higher OP concentrations (1000 and 2000 nM) progressively increased testosterone levels when compared to control (Fig. 4).

3.7. Effect of exposure to Triton X-100 or sodium cholate on testosterone formation

Cultured Leydig cells were exposed to Triton X-100 (10–2000 nM) or sodium cholate (10–2000 nM) alone for 4 h. Following treatment, media were collected for quantitation of testosterone. In contrast to OP, the highest concentrations of Triton X-100 reduced testosterone levels from 5.1 ng ± 0.3 ng/10^5cells (control) to 3.5 ng ± 0.2 ng/10^5 cells (Fig. 5, panel A); however, sodium cholate was without effect on testosterone levels (Fig. 5, panel B).

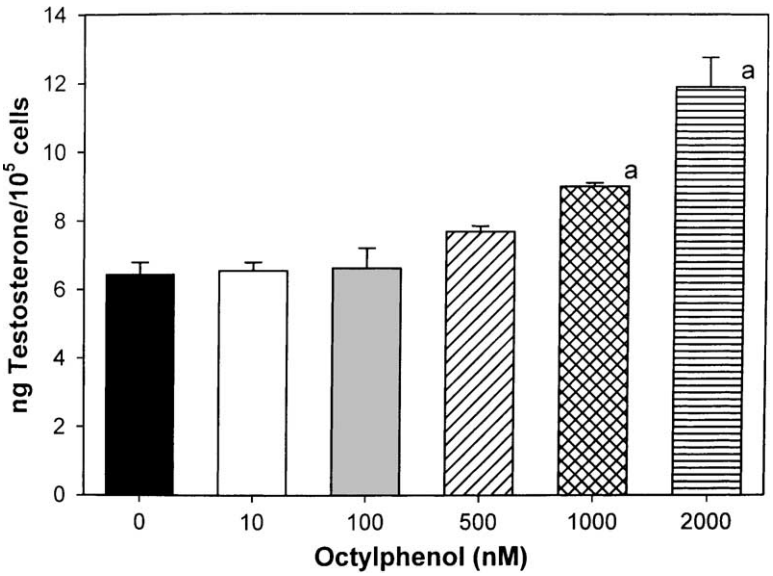

Fig. 4. Inclusion of 5α-reductase inhibitor on effect of octylphenol on testosterone formation of cultured Leydig cells. On day 1 of culture, fresh media containing 1 μM 4-MOPC were added. After 15 min, increasing concentrations of OP were added, and cells were cultured for 4 h. Each value represents the mean ± S.E.M. of 4 samples from a single experiment, and these results are representative of at least three separate experiments. a, $P < 0.05$ when compared with control.

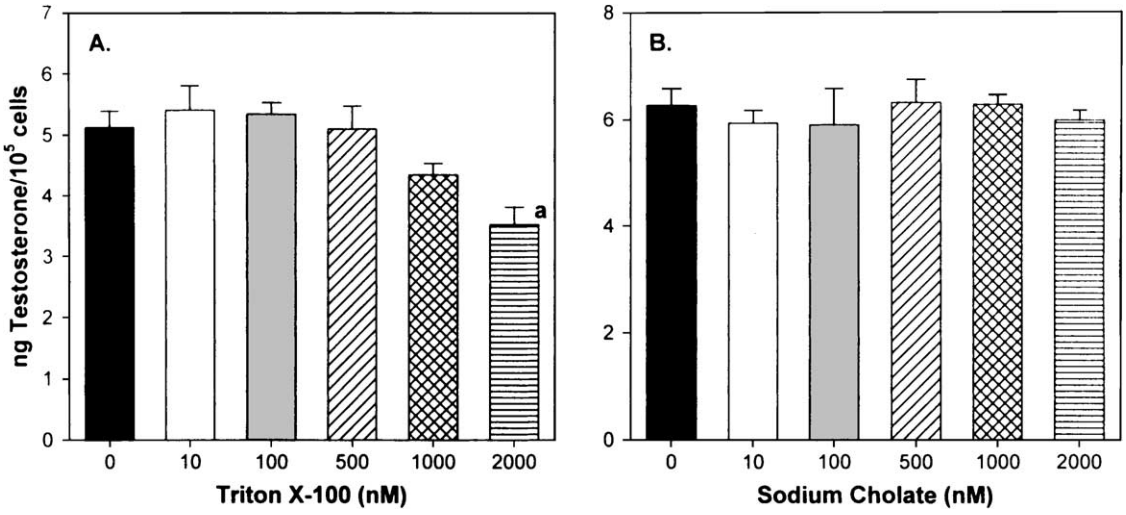

Fig. 5. Effect of Triton X-100 or sodium cholate on testosterone formation by cultured Leydig cells. Leydig cells were exposed to increasing concentrations of Triton X-100 (panel A) or sodium cholate (panel B) for 4 h. Each value represents the mean ± S.E.M. of four samples from a single experiment, and these results are representative of at least three separate experiments. a, $P < 0.05$ when compared to appropriate control.

4. Discussion

The current studies evaluated the possible mechanism(s) to explain the constitutive increase in testosterone levels of cultured Leydig cells from young adult rat testes following exposure to OP. This increase was not due to the entry of extracellular calcium into Leydig cells or to the release of calcium from intracellular stores. The increase in testosterone was not associated with any change in intracellular cAMP levels, and it did not involve the binding of OP to the estrogen receptor α or β. Furthermore, the increase in testosterone was not the consequence of inhibiting Leydig cell 5α-reductase activity which would result in the accumulation of testosterone, nor was the effect due to a potential detergent property of OP. Although these results did not identify signaling pathways that are activated to explain the constitutive increase in testosterone following exposure to OP, they emphasize the potential varied actions of this chemical on Leydig cell androgen formation.

Leydig cell androgen biosynthesis is regulated primarily by luteinizing hormone (LH), and the primary intracellular second messenger that mediates its effect is cAMP [37]. In the current studies, we observed no change in intracellular cAMP levels over a period of 0.5–24 h following exposure to OP alone, suggesting that changes in cAMP levels do not mediate the effects of OP on basal testosterone formation.

Optimal androgen production by Leydig cells requires extracellular calcium [38], and treatment of cells with LH increases intracellular calcium levels [39,40]. Our current results, where exposure to OP alone in media lacking calcium and/or containing the membrane-permeable calcium chelator, BAPTA, still increased testosterone levels, suggest that neither the entry of extracellular calcium or its release from intracellular stores mediates this effect of OP.

OP has been reported to exhibit weak estrogenic activity in many testing protocols [13–16]; therefore, whether the increase in testosterone levels following exposure to OP alone is mediated by the binding of OP to estrogen receptors was evaluated. The inability of the concomitant exposure to the pure estrogen antagonist, ICI, to block the stimulatory effects of OP suggests that this action of OP does not involve binding to either estrogen receptor α or β. Furthermore, it was reported previously that exposure of cultured adult Leydig cells to 17β-estradiol alone for 4 or 24 h had no effect on testosterone formation [25].

Immature rat Leydig cells exhibit high 5α-reductase activity [33,34] which effectively "metabolizes" synthesized testosterone to DHT and to hydroxylated metabolites of DHT (3α- and 3β-diol) [35]. After about day 40 following birth, 5α-reductase activity declines progressively to reach very low levels by about day 60 [33] and testosterone becomes the predominant androgen. Thus, if there were sufficient residual 5α-reductase activity in Leydig cells from young adult rats, inhibition of this enzyme by OP could result in elevated testosterone levels [36]. The observation that testosterone levels increased progressively following concomitant exposure to OP and the 5α-reductase inhibitor, 4-MOPC, suggests that OP does not alter testosterone formation by inhibiting Leydig cell 5α-reductase activity.

The parent compound of OP, octylphenol ethoxylate, exhibits detergent qualities [11]. Whether OP exhibits comparable detergent effects on cell membranes is not known. To evaluate whether the ability of OP alone to increase testosterone formation is due to its detergent characteristics on Leydig cell membranes, to which steroidogenic enzymes are attached, the effects of the detergents Triton X-100 (which is identical to OP, but it contains an average of 9.5 ethylene oxide subunits attached to the phenolic ring) or sodium cholate on testosterone formation were evaluated. For solubilization of membrane-associated proteins, typically 0.1% of Triton X-100 [41] and 0.8% of sodium cholate [42] are used. These concentrations are more than 800- and 9000-fold higher, respectively, than the highest concentration of OP used in the current studies. Neither Triton X-100 or sodium cholate increased testosterone levels in the present studies, suggesting that these effects of OP are not due to its detergent characteristics.

Although LH, through the activation of the cAMP and protein kinase A (PKA) signaling pathway, is the primary hormone that regulates Leydig cell testosterone formation [37], other hormones or growth factors, using alternative signaling pathways (which may interact with the cAMP or other signaling pathways) have been shown to affect testosterone biosynthesis. For example, insulin or insulin-like growth factor-1 (IGF-1) [43], epidermal growth factor (EGF) [44], luteinizing hormone releasing hormone (LHRH) [45] and atrial natriuretic factor (ANF) [46], either alone or with LH/hCG, were reported to increase testosterone biosynthesis in rat or mouse Leydig cells. Prolactin has been reported to both stimulate or inhibit testosterone formation, with the direction of response being dependent on hormone dosage and animal age [47,48]. Insulin, IGF-1 and EGF exert their effects on responsive cells by binding to specific receptors having intrinsic tyrosine kinase activity, which, in turn, activate the Ras/Raf/MAPK signaling pathway [49]. The actions of LHRH on Leydig cells are thought to be mediated through an increase in phosphatidyl inositol turnover [50], while that of ANF is thought to be mediated through increases in cellular cGMP levels [46]. With respect to prolactin actions, following its binding to specific receptors, cytoplasmic tyrosine kinase(s) such as Janus kinase(s) (JAKs) are proposed to be recruited to the receptor and activated. These JAKs, in turn, phosphorylate signal transducers and activators of transcription (STATs), which are able to activate transcription [51]. It is possible that exposure to OP alone activates an alternative signaling pathway(s) to enhance testosterone formation, and this effect is masked when hCG is included in the incubation medium [25]. It should be emphasized that in the presence of hCG, exposure to OP inhibited testosterone formation by rat Leydig cells, with the main locus of action occurring between the enzymatic steps converting cholesterol to androstenedione [25].

The increase in testosterone following stimulation by LH is proposed to involve activation of PKA by cAMP, phosphorylation of cAMP response element-binding protein (CREB) by PKA and the binding of phosphorylated CREB to the promotor element(s) of cAMP-regulated genes (proteins/enzymes involved in testosterone biosynthesis). However, in addition to hormone-activated functions, CREB plays a role in basal transcription [52], and CREB contains two separate and independently acting activation domains, a constitutive activation domain (CAB) responsible for basal activation and a kinase inducible domain that mediates activation in response to PKA [53]. Thus, it is possible that OP or similar compounds could enhance CREB binding to CAB to increase basal testosterone biosynthesis.

In addition to activating signals, inhibitory signals can modulate cellular functional status [54]. In this regard, the net functional activity of a cell could be enhanced by blocking one or more inhibitory signals. Whether the increase of testosterone formation following exposure to OP alone is

mediated by blocking potential inhibitory signals or by activating an alternative signaling pathway(s), acting alone or intersecting with other signaling pathway(s), remains to be determined.

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