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The reported active metabolite of methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, inhibits testosterone formation by cultured Leydig cells from neonatal rats

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Abstract

Methoxychlor (MC) is an insecticide that is presently used on agricultural crops, especially after the ban on the use of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) in the United States. Following administration in vivo, MC is converted to 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is thought to be the active agent. However, both MC and HPTE have been reported to have weak estrogenic and antiandrogenic activities, and they are thought to exert their potential adverse (endocrine disruptive) effects through the estrogen and androgen receptors, respectively. In a recent study, HPTE was shown to inhibit both basal and hCG-stimulated testosterone production by cultured Leydig cells from immature and adult rats, and these effects were reported to be mediated through the estrogen receptor. Because fetal Leydig cells represent a separate population from adult Leydig cells and many of the reported adverse actions of endocrine disruptors are thought to have their effects during gestational exposure, the present studies examined the effects of HPTE on testosterone formation by cultured fetal Leydig cells from neonatal rats to determine whether these cells are sensitive to HPTE. Our studies demonstrated that HPTE inhibited both basal and hCG-stimulated testosterone formation in a dose-dependent manner. Significant declines in testosterone were observed at about 100 nM HPTE, and this effect was detected as early as 1 h after exposure. The main effects of HPTE appeared to be localized to the cholesterol side-chain cleavage step which converts cholesterol to pregnenolone. In addition, this effect did not appear to be mediated through the estrogen receptor as a weak estrogen or the androgen receptor as an antiandrogen, which are the currently proposed modes of action of MC and HPTE.

Keywords: HPTE; Neonatal Leydig cell; Testosterone

1. Introduction

It has been proposed that environmental/occupational exposure to endocrine-disrupting chemicals has altered the normal functioning of the endocrine system of humans and various wildlife species and that this has resulted in adverse reproductive effects [1,2]. Several reproductive changes ascribed to these exposures over the past 40–50 years in males include: (1) a world-wide decline in semen quality [3], and (2) an increase in the prevalence of cryptorchidism [4], hypospadias [5] and testicular cancer [6]. However, the causal association between exposure to endocrine-disrupting chemicals and the increased incidence in male reproductive disorders is controversial and not universally accepted [7].

Methoxychlor (2,2-bis(*p*-methylphenyl)-1,1,1-trichloroethane; MC) is an insecticide currently used on several agricultural crops, especially following the ban of 2,2-bis(*p*-chlorophenyl)-1,1,l-trichloroethane (DDT) use in the United States in 1972. Its advantages over DDT are that it is more rapidly metabolized and excreted in mammals [8], it is

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acutely less toxic than DDT [9] and it is biodegradable [8]. In vivo administration of MC to immature female rats increases uterine weight [10] and stimulates uterine omithine decarboxylase activity [11], which are both estrogen-associated responses. Following in vivo administration in rats, MC is metabolized in the liver to 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is thought to be the active agent because it binds with higher affinity to the estrogen receptor (ER) than MC [12]. However, recent studies suggest that MC has weak intrinsic estrogenic activity [13] and that it binds to both ER isoforms (ERα and ERβ) [14]. With respect to differences in the binding characteristics of HPTE to ERα and ERβ, HPTE acted as an ERα agonist in human hepatoma cells (HepG2), but acted mainly as a competitive antagonist with ERβ [15]. In addition to estrogenic activities, MC has been reported to exhibit antiandrogenic properties following in vivo exposure to male rats [16]. In HepG2 cells transiently transfected with the human androgen receptor (AR) and an androgen-responsive reporter, both MC and HPTE were antagonistic, although HPTE was about an order of magnitude more potent [17]. Thus, with respect to the potential reproductive effects of MC and HPTE, the steroid receptor type (ER or AR) and sub-type (ERα and ERβ) appear to affect the nature of the response. Although blocking AR-mediated actions offers one mechanism of action for explaining the antiandrogenic effects of MC and HPTE, other potential mechanisms include inhibiting testosterone biosynthesis by Leydig cells or inhibiting the conversion of testosterone to dihydrotestosterone (DHT) (inhibition of 5α-reductase activity) in DHT-sensitive tissues.

During fetal development in the rat, Leydig cells appear and begin to secrete testosterone on about day 15 of gestation [18,19]. Although testosterone secreted by fetal Leydig cells is essential for the differentiation of the reproductive ductal structures, 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 2–3 weeks after birth and remain steroidogenically competent [20,21]. After this period, they regress and are replaced by an adult-type Leydig cell [22,23]. Accordingly, Leydig cells isolated during the first 2 weeks after birth represent the fetal generation of cells.

A recent study reported that HPTE can directly inhibit both basal and hCG-stimulated testosterone biosynthesis by cultured Leydig cells from immature and adult rats and that this response was mediated through the ER [24]. It has not been established whether HPTE has similar effects on fetal Leydig cells. Because of the important role of androgen in regulating male reproductive development during the fetal period and because many of the reported adverse effects of endocrine-disrupting chemicals in males are expected to occur during gestational exposure, the current studies examined the effects of HPTE on testosterone formation by cultured fetal Leydig cells from neonatal rats.

2. Materials and methods

2.1. Animals

Neonatal (1–3 days old) male Sprague–Dawley rats (Hla: (SD)CVF) were purchased from Hilltop Lab Animals Inc., Scottdale, PA, USA. They were housed in polycarbonate shoebox cages (one litter of 10–12 pups with a nursing mother per cage) and exposed to a 12 h light and 12 h dark cycle. The bedding material consisted of a mixture of Alpha-dri (Shepard Specialty Paper, Watertown, TN, USA) and Beta Chip (Virgin hardwood chips from NEPCO, Warrenburg, NY, USA). The mothers received Purina rat chow (R-M-H 3500 with 5% fat content) and 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 Care and Use Committee.

2.2. Reagents

Collagenase (Sigma Blend Type L), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNase I), 22(*R*)-hydroxycholesterol, 25-hydroxycholesterol, 5-pregnen-3β-ol-20-one (pregnenolone), 4-pregnen-3,20-dione (progesterone), 4-androsten-3,17-dione (androstenedione), dimethylsulfoxide (DMSO), 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), and neutral alumina were purchased 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. Chloroform was from Fisher Scientific, Pittsburgh, PA, USA. Dulbecco's Modified Eagle Medium (DMEM) without phenol red, F-12 nutrient mixture (F-12) without phenol red, medium 199 (Med 199), Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, sodium bicarbonate, soybean trypsin inhibitor, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY, USA. [2,3,6,7-^{3}H(N)]-Testosterone (specific activity ∼100 Ci/mmol), 25-[26,27-^{3}H]-hydroxycholesterol (specific activity ∼80 Ci/mmol), and ^{14}C-isocaproic acid were from Perkin-Elmer Life Sciences, Boston, MA, USA. Testosterone and 17β-estradiol were from Steraloids, Wilton, NH, USA. Percoll was from Pharmacia, Piscataway, NJ, USA. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a derivative of the antioxidant α-tocopherol) was from Aldrich, Milwaukee, WI, USA. 2,2,Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (99% pure) was from Cedra Corp., Austin, TX, USA. Vinclozolin was from Crescent Chemical Co., Hauppage, NY, USA. 2-[[(3,5-Dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3′:5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) were gifts from the EPA/NHEERL, Research Triangle Park, NC, USA, through Dr. William Kelce, Pharmacia Corp., Kalamazoo, MI, USA. 4-Hydroxyflutamide was a

gift from Schering-Plough Research Corp., Kenilworth, NJ, USA. ICI 182,780 was a gift from Dr. A.E. Wakeling, Zeneca Pharmaceuticals, Cheshire, UK. Human chorionic gonadotropin (hCG, CR-127, 14,900 IU/mg) was a gift from NIDDKD, Bethesda, MD, USA.

2.3. Isolation and culture of Leydig cells

Animals were 6–7 days old when sacrificed by placing them in a chamber saturated with CO_2. For most studies, testes from 8 to 10 litters of male pups were collected, pooled, decapsulated and digested in 0.25 mg/ml collagenase in Med 199 + 0.1% BSA and 10 μg/ml DNase I for 30–40 min at 37 °C. The dispersed interstitial cells were layered over a 60% Percoll gradient and centrifuged at ∼12,000 × *g* for 1 h at 4 °C. Cells localizing between densities of 1.052 and 1.068 g/ml were isolated as described previously [25]. These cells represented ∼20% neonatal (fetal) Leydig cells based on positive staining for 3β-HSD) activity [26].

Leydig cells were suspended in a 1:1 mixture of DMEM/F-12 lacking phenol red and 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 into 1.6 cm diameter, 24-well Costar culture plates. To each well was added 10^5 cells in 1 ml of medium, and cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 33 °C as described previously [25], except fresh medium (now lacking BSA) was added ∼20 h rather than 2 days after plating. Treatments were initiated right after media change in all studies except for the P4SOscc activity study where cells were exposed to HPTE on the day of plating. HPTE was dissolved in DMSO, and the final DMSO concentration in all treatment groups was 0.1%. This concentration of DMSO did not affect testosterone formation by neonatal Leydig cells. The concentrations of HPTE used did not affect cell viability as judged by the ability of exposed cells to exclude trypan blue dye, cell morphology and continued attachment of cells to culture wells. In experiments where vinclozolin, M1 or M2 alone were added, they were dissolved in DMSO, and the final DMSO concentration in all treatments groups was 0.1%. When 22(*R*)-hydroxycholesterol, pregnenolone, progesterone, androstenedione, ICI, Trolox, or 4-hydroxyflutamide were added, they were dissolved in ethanol, and the final ethanol concentration in all treatment groups was 0.1%. This concentration of ethanol did not adversely affect Leydig cell testosterone formation or cell viability as assessed for cells exposed to DMSO.

2.4. Quantitation of testosterone by radioimmunoassay (RIA)

Testosterone was quantitated by RIA directly from the medium as described previously [27]. None of the chemicals tested in this study interfered with the RIA at the concentrations tested.

2.5. Measurement of P450 cholesterol side-chain cleavage activity

P450 cholesterol side-chain cleavage activity (P4SOscc) of cultured Leydig cells from neonatal rats was determined by measuring the conversion of 25-[26,27-^{3}H]-hydroxycholesterol to pregnenolone and ^{3}H-labeled water-soluble side-chain by following a previously described procedure [28] with slight modifications. In brief, following the exposure of cultured cells (5×10^5/ml medium) to varying concentrations of HPTE alone for 24 h, fresh medium (0.5 ml) containing substrate (0.5 μCi, 5 μM) was added to each well, and the cells were incubated for 4 h at 33 °C in an atmosphere of 95% air and 5% CO_2. Reactions were stopped by adding 50 μl of 1N NaOH to each well. The contents of each well were transferred to 7 ml borosilicate glass vials, and each well was washed with 1.05 ml phosphate-buffered saline (PBS). The PBS washes were transferred to the vials containing the corresponding incubation media, and the contents were extracted with 4 ml of chloroform. After separation of the two phases, 0.8 ml of the upper aqueous phase containing the water-soluble ^{3}H-labeled side-chain was removed and placed in a 5 ml borosilicate glass culture tube containing 0.25 g neutral alumina, which adsorbs any contaminating substrate. The lower organic phase contains unmetabolized ^{3}H-labeled substrate and unlabeled steroid product(s) (e.g., pregnenolone). A known amount of ^{14}C-isocaproic acid, was processed in separate wells in a similar manner to estimate recoveries, which averaged 95–100%. The aqueous phase and the neutral alumina were mixed, and the tubes were centrifuged at ∼3000 rpm for 20 min to settle the neutral alumina. An aliquot of the aqueous phase was removed and counted using Ecolite as the scintillation fluid.

2.6. Statistical analysis

Data are expressed as the mean ± S.E.M. and analyzed by ANOVA. Differences among treatment means were determined using Student–Newman–Keuls' test. A *P*-value of ≤0.05 was considered statistically significant. For the time studies, data were analyzed using Student's *t*-test.

3. Results

3.1. The effect of HPTE on basal or hCG-stimulated testosterone

Studies were initiated to determine whether testosterone formation by fetal Leydig cells in response to HPTE was sensitive to the time of exposure (4 h versus 24 h), the dose of HPTE added and to the absence or presence of hCG. The basal testosterone level following 4 h of culture was 0.20 ± 0.01 ng testosterone/10^5 cells. In response to exposure to increasing concentrations of HPTE (0, 100, 500 or 1000 nM) alone for 4 h, a significant decline in testosterone was observed at

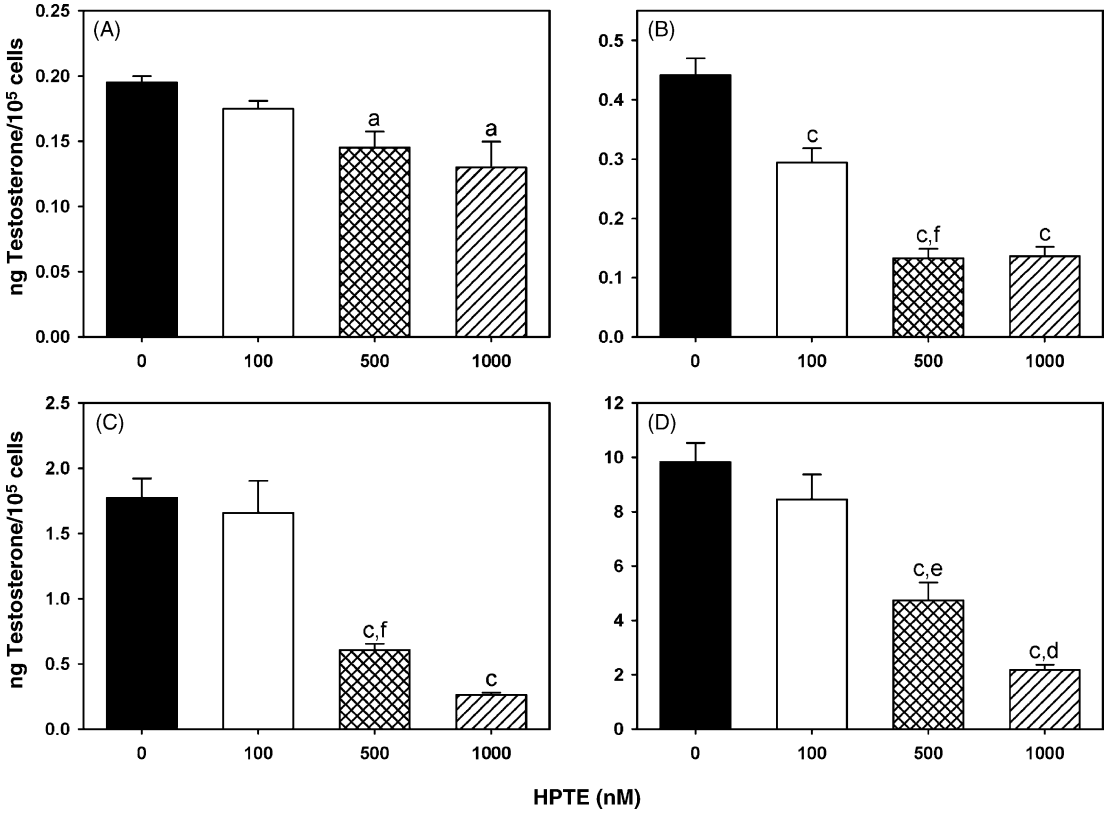

Fig. 1. Effect of HPTE on basal or hCG-stimulated testosterone. Leydig cells were exposed to HPTE alone (0, 100, 500 or 1000 nM) for 4 h (panel A) or 24 h (panel B) or to HPTE (0, 100, 500 or 1000 nM) and 10 IU/ml hCG for 4 h (panel C) or 24 h (panel D). Each treatment group represents the mean ± S.E.M. of four separate samples from a single experiment, and these results are representative of at least three separate experiments. (a, c) $P < 0.05$ and 0.001, respectively, when compared to the appropriate control group receiving 0.1% DMSO (vehicle for HPTE) alone; (d–f) $P < 0.05$, 0.01, and 0.001, respectively, when compared to the appropriate immediate lower HPTE concentration.

500 nM (74% of control; Fig. 1, panel A). At 1000 nM HPTE, the testosterone level was 67% of control. Leydig cells cultured for 24 h in the absence of hCG produced 0.44 ± 0.03 ng testosterone/10^5 cells. When neonatal Leydig cells were exposed for 24 h to increasing concentrations of HPTE, a significant decline in testosterone was observed at 100 nM HPTE (66% of control; Fig. 1, panel B), and progressive declines were observed at higher concentrations. At 1000 nM HPTE, the testosterone level was 31% of control. Leydig cells produced 1.77 ± 0.15 ng testosterone/10^5 cells following culture for 4 h in the presence of 10 mIU/ml hCG (∼9-fold increase over 4 h basal level). In response to exposure to 10 mIU/ml hCG and HPTE (0, 100, 500 or 1000 nM) for 4 h, a significant decline in testosterone level (34% of control; Fig. 1, panel C) was observed at 500 nM. Testosterone level declined further to 15% of control at 1000 nM HPTE. Leydig cells produced 9.83 ± 0.70 ng testosterone/10^5 cells following culture for 24 h in the presence of 10 mIU/ml hCG (∼22-fold increase over 24 h basal level). Exposure of neonatal Leydig cells to hCG and increasing HPTE concentrations for 24 h resulted in a decline in testosterone (48% of control; Fig. 1, panel D) at 500 nM HPTE. The testosterone level declined further to 22% of control at 1000 nM HPTE.

Studies also were conducted to determine whether HPTE inhibits testosterone formation prior to the 4 h exposure period. Because basal testosterone levels in neonatal Leydig cells are low during incubations less than 4 h, the effect of 1000 nM HPTE on 10 mIU/ml hCG-stimulated testosterone was evaluated following exposure for 1, 2 and 4 h. A significant decline in testosterone was observed following 1 h of exposure (64% of control; Fig. 2). Testosterone levels decline further to 44 and 14% of control following exposures for 2 and 4 h, respectively (Fig. 2). Collectively, these results demonstrate that HPTE inhibits both basal and hCG-stimulated testosterone levels at concentrations as low as 100 nM, and that inhibition can be observed following 1 h of exposure.

3.2. The effect of HPTE on 8-Br-cAMP-stimulated testosterone

Because HPTE inhibited both basal and hCG-stimulated testosterone formation by cultured neonatal Leydig cells, it was evaluated whether the primary effects of HPTE occurred before or after the formation of cAMP. In response to exposure to 1 mM 8-Br-cAMP and HPTE (0, 100, 500 and

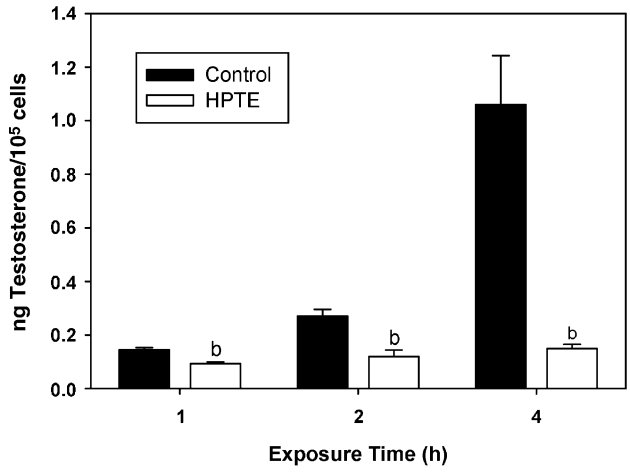

Fig. 2. Effect of exposure time to HPTE on hCG-stimulated testosterone. Leydig cells were exposed to 10 mIU/ml hCG and 0.1% DMSO (control) or with 10 mIU/ml hCG plus 1000 nM HPTE for 1, 2 or 4 h. Each HPTE-exposed group was compared with the corresponding time control. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (b) $P < 0.01$ when compared with the appropriate control group for each exposure time.

1000 nM) for 4 h, a significant decline in testosterone was observed at 500 nM HPTE (47% of control; Fig. 3, panel A). The testosterone level declined further to 14% of control at 1000 nM HPTE. When neonatal Leydig cells were exposed to the same concentrations of HPTE and 1 mM 8-Br-cAMP for 24 h, a significant decline in testosterone was observed at 100 nM (62% of control; Fig. 3, panel B). Testosterone levels declined progressively to 38 and 18% of control at 500 and 1000 nM HPTE, respectively. These results suggest that the main actions of HPTE occur after the formation of cAMP in neonatal Leydig cells.

3.3. The effect of exposure to HPTE alone for 24 h on the subsequent conversion of steroid precursors to testosterone

In order to determine whether exposure to HPTE inhibits a steroidogenic enzyme(s) involved in the conversion of cholesterol to testosterone, neonatal Leydig cells were exposed to HPTE (0, 100, 500 and 1000 nM) alone for 24 h. Next, fresh medium containing 10 μM of 22(*R*)-hydroxycholesterol, 10 μM pregnenolone, 10 μM progesterone or 10 μM androstenedione was added to each well. Following incubation for 4 h with each substrate, the formation of testosterone was measured which provides an indirect measure of P450scc, 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD), P450 17α-hydroxylase/17,20 lyase (P450c17) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities, respectively. Following the addition of 22(*R*)-hydroxycholesterol, a significant decline in testosterone was observed in Leydig cells exposed to 100 nM HPTE (declining to 50% of control; Fig. 4, panel A). The conversion of 22(*R*)-hydroxycholesterol to testosterone declined further to 25 and 24% of control in cells exposed to 500 and 1000 nM HPTE, respectively. In contrast, the conversion of pregnenolone (Fig. 4, panel B), progesterone (Fig. 4, panel C) or androstenedione (Fig. 4, panel D) to testosterone was unaffected by prior exposure to HPTE, suggesting that among the enzymes converting cholesterol to testosterone, only P450scc activity is inhibited by HPTE. These studies were repeated in Leydig cells exposed to increasing HPTE concentrations and

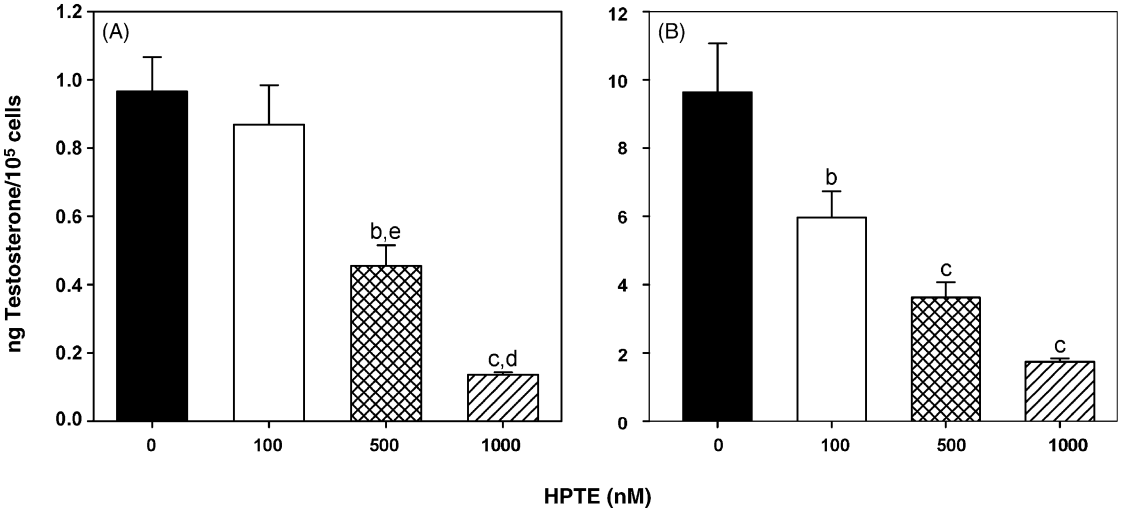

Fig. 3. Effect of HPTE on 8-Br-cAMP-stimulated testosterone. Leydig cells were exposed to HPTE alone (0, 100, 500 or 1000 nM) and 1 mM 8-Br-cAMP for 4 h (panel A) or 24 h (panel B). Control cells received 0.1% DMSO (vehicle for HPTE). Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (b, c) $P < 0.01$ and 0.001, respectively, when compared with the appropriate control group; (d, e) $P < 0.05$ and 0.01, respectively, when compared with the immediate lower HPTE concentration of the same exposure time.

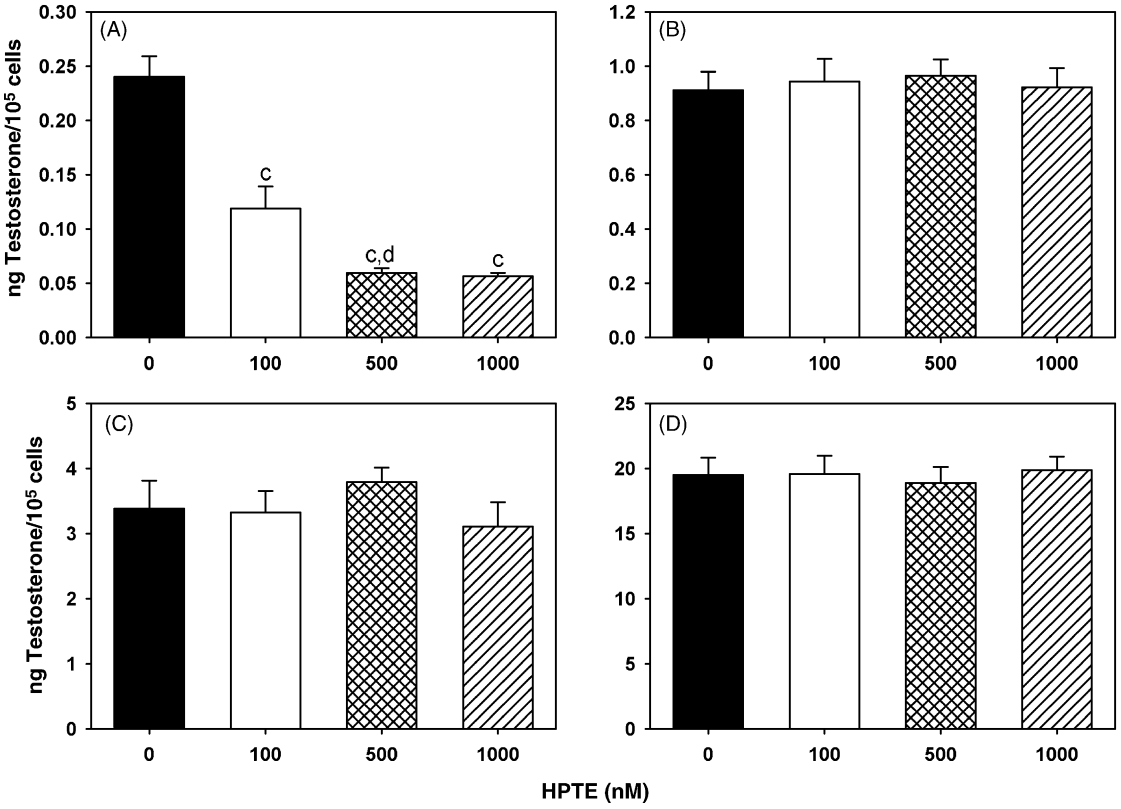

Fig. 4. Effect of initial exposure to HPTE on subsequent conversion of steroid substrates to testosterone. Leydig cells were exposed to HPTE (0, 100, 500 or 1000 nM) alone for 24 h. Control cells received 0.1% DMSO (vehicle for HPTE). Next, fresh medium containing 10 μM 22(*R*)-hydroxycholesterol, 10 μM pregnenolone, 10 μM progesterone or 10 μM androstenedione was added to each well, and the formation of testosterone after incubation for 4 h was determined (panels A–D, respectively). Each treatment group represents the mean ± S.E.M. of four separate samples from a single experiment, and these results are representative of at least three separate experiments. (c) $P < 0.001$ when compared with the appropriate control group; (d) $P < 0.05$ when compared with the immediate lower HPTE concentration of the appropriate treatment group.

10 mIU/ml hCG for 24 h, and similar results were observed (data not shown).

3.4. The effect of HPTE on P450scc activity

To confirm that HPTE inhibits P450scc activity, neonatal Leydig cells were exposed to HPTE (0, 40, 200 and 1000 nM) alone for 24 h. Thereafter, fresh medium containing 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) was added to each well, and the cells were incubated for 4 h to quantitate the release of ^{3}H-labeled side-chain into the medium. Control cells released 3.40 ± 0.10 ng side-chain over 4 h per 5×10^5 cells (Fig. 5). In response to exposure to 40 nM HPTE, P450scc activity declined to 72% of control. Exposure to 200 and 1000 nM HPTE resulted in further progressive declines in P450scc activity to 57 and 46% of control, respectively.

3.5. The effects of ICI and HPTE on testosterone formation

Neonatal Leydig cells were exposed concomitantly to HPTE (0, 100, 500 or 1000 nM), 5 μM ICI (a "pure"

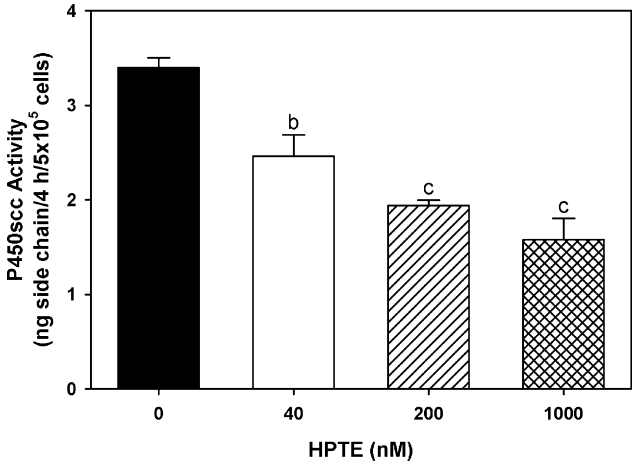

Fig. 5. Effect of HPTE on P450scc activity of Leydig cells. Leydig cells were exposed to HPTE (0, 40, 200 or 1000 nM) alone for 24 h. Following exposure, P450scc activity (ng of side-chain released over 4 h per 5×10^5 cells) was estimated on intact cells. Each treatment group represents the mean ± S.E.M. of four separate samples from a single experiment, and these results are representative of at least three separate experiments. (b, c) $P < 0.01$ and 0.001, respectively, when compared to control.

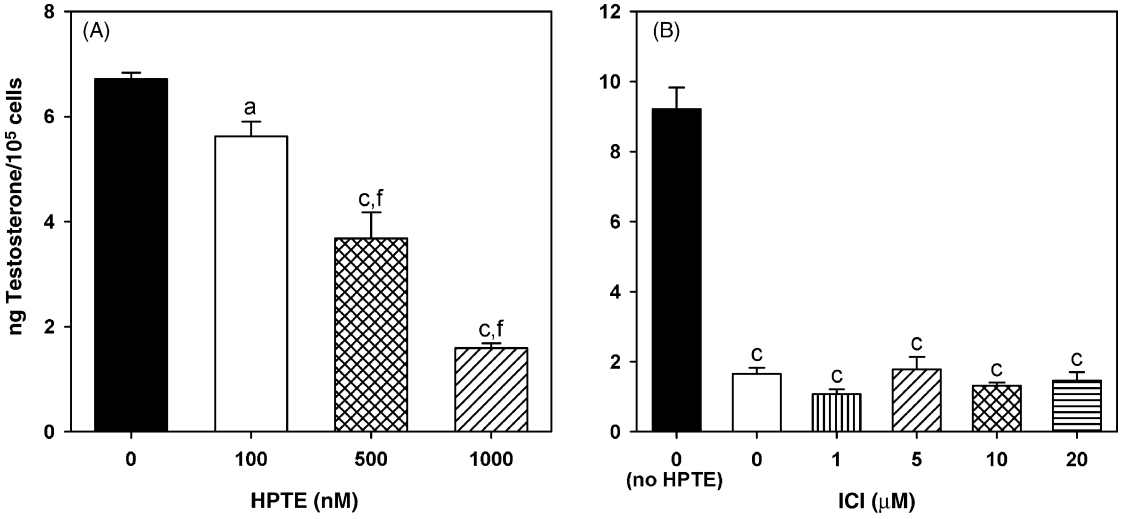

Fig. 6. Effect of ICI on HPTE effects on Leydig cell testosterone. Leydig cells were exposed to 10 mIU/ml hCG and 5 μM ICI (all treatment groups), and HPTE (0, 100, 500 or 1000 nM) (panel A) or to 10 mIU/ml hCG (all treatment groups) without or with 1000 nM HPTE and without or with ICI (1–20 μM) (panel B) for 24 h. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (a, c) $P < 0.05$ and 0.001, respectively, when compared with the appropriate control group; (f) $P < 0.001$ when compared with the immediate lower HPTE concentration group.

estrogen receptor antagonist [29,30] and 10 mIU/ml hCG for 24 h to assess whether the actions of HPTE were mediated through ERα or ERβ. Under these conditions, 100 nM HPTE reduced testosterone to 84% of control, and higher concentrations of HPTE progressively reduced testosterone levels to 24% of control at 1000 nM (Fig. 6, panel A). To test whether both lower or higher ICI concentrations similarly were without effect on the inhibitive effects of HPTE on testosterone formation, neonatal Leydig cells were exposed to 10 mIU/ml hCG alone or 10 mIU/ml hCG + 1000 nM HPTE and increasing concentrations of ICI (0, 1–20 μM). Under these conditions, none of the ICI concentrations reversed the effects of HPTE (Fig. 6, panel B). The results suggest that the actions of HPTE are not mediated through the ERα or ERβ pathway.

3.6. The effects of Trolox on the inhibitive effects of HPTE

Studies were conducted to determine whether the addition of HPTE to neonatal Leydig cells caused excess electron leakage, which could result in the formation of free radicals. These free radicals could cause lipid peroxidation and damage to 450scc localized in mitochondria. To evaluate this possibility, neonatal Leydig cells were treated for 24 h with 10 mIU/ml hCG alone or with 10 mIU/ml hCG and 1000 nM HPTE concomitantly with increasing concentrations of water-soluble Vitamin E derivative, Trolox (0–100 μM). In the presence of hCG alone, neonatal Leydig cells produced 7.55 ± 0.82 ng testosterone/10^5 cells after 24 h (Fig. 7). The addition of 1000 nM HPTE to hCG-stimulated cells lowered the testosterone level to 18% of control. The inclusion of increasing concentrations of Trolox did not reverse the pattern of HPTE-induced inhibition.

3.7. The effect of hydroxyflutamide or vinclozolin and its metabolites on testosterone formation

Previous studies have shown that HPTE exhibits antiandrogenic properties [16]. To evaluate whether HPTE acted as an antiandrogen to inhibit testosterone formation, neonatal Leydig cells were exposed to 10 mIU/ml hCG and increasing concentrations of the antiandrogen, hydroxyflutamide (0,

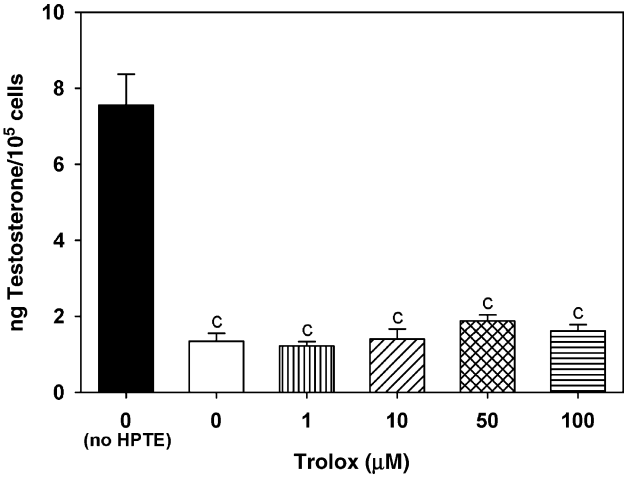

Fig. 7. Effect of Trolox on the effect of HPTE on Leydig cell testosterone. Leydig cells were exposed to 10 mIU/ml hCG, without or with 1000 nM HPTE and without or with Trolox (1–100 μM) for 24 h. Control cells received 0.1% DMSO (vehicle for HPTE) and 0.1% ethanol (vehicle for Trolox). Each treatment group represents the mean ± S.E.M. of four determinations from a single experiment, and these results are representative of at least three separate experiments. (c) $P < 0.001$ when compared with control (no HPTE group). Trolox-treated groups were not different than no Trolox control group receiving HPTE.

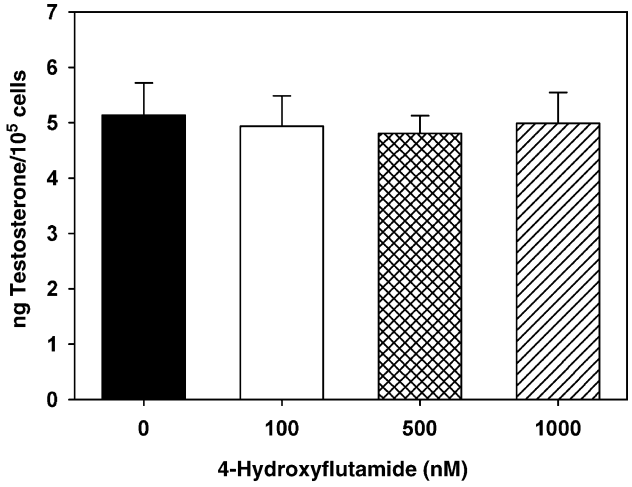

Fig. 8. Effect of hydroxyflutamide on Leydig cell testosterone. Leydig cells were exposed to 10 mIU/ml hCG and hydroxyflutamide (0, 100, 500 or 1000 nM) for 24 h. Hydroxyflutamide was dissolved in ethanol, and all treatment groups contained 0.1% of the vehicle as a final concentration. Each treatment group represents the mean ± S.E.M. of four determinations from a single experiment, and these results are representative of at least three separate experiments.

100, 500 or 1000 nM), for 24 h. Under these conditions, hydroxyflutamide had no effect on testosterone levels (Fig. 8). These results suggest that the inhibition of testosterone formation by HPTE in neonatal Leydig cells is not due to its antiandrogenic properties. Vinclozolin is an agricultural fungicide which is currently used in both the United States and Europe. In vivo, it is metabolized to 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid and to 3′,5′-dichloro-2-hydroxy-2-methyl-but-3-enanilide [31]. The parent compound and its two metabolites are reported to have antiandrogenic activity, with the order of potency: M2 > M1 > vinclozolin [32]. We evaluated the effects of vinclozolin, M1 or M2 on 10 mIU/ml hCG-stimulated testosterone formation by neonatal Leydig cells following 24 h of exposure. Under these conditions, vinclozolin (100–1000 nM), M1 (100–1000 nM) or M2 (100–1000 nM) (Fig. 9, panels A–C, respectively) had no effect on hCG-stimulated testosterone formation.

3.8. Recovery of neonatal Leydig cell testosterone formation following HPTE exposure

Because of the time- and dose-dependent declines in testosterone formation of neonatal Leydig cells following exposure to HPTE in these studies, we evaluated whether testosterone formation recovers following this inhibition. In response to 10 mIU/ml hCG alone for 4 h, the testosterone level was 1.06 ± 0.18 ng/10^5 cells (Fig. 10, panel A). Concomitant inclusion of 1000 nM HPTE reduced the testosterone level to 14% of control. Following exposure, fresh medium containing 10 mIU/ml hCG was added to all wells, and the cells were cultured for 24 h. Control cells not previously exposed to HPTE produced 1.36 ± 0.26 ng testosterone/10^5 cells; however, cells previously exposed to HPTE produced 0.89 ± 0.06 ng testosterone (65% of control, not significantly different from control; Fig. 10, panel B). Following the initial 24 h recovery period, fresh medium containing 10 mIU/ml hCG was added to all wells for a second 24 h recovery period. Control cells produced 0.72 ± 0.14 ng testosterone over this period, while cells previously exposed to HPTE produced 0.83 ± 0.08 ng testosterone (116% of control, not significantly different from control). These studies demonstrate that over 1–2 days following exposure to HPTE, testosterone biosynthetic capacity fully recovers when hCG is included in the culture medium.

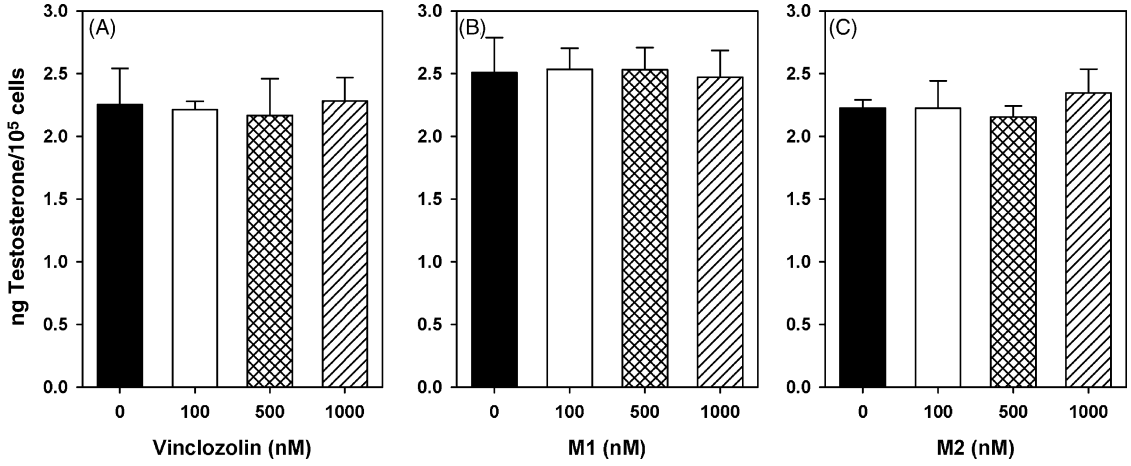

Fig. 9. Effect of vinclozolin or its active metabolites, M1 and M2, on Leydig cell testosterone. Leydig cells were exposed to 10 mIU/ml hCG and vinclozolin (0, 100, 500 or 1000 nM) (panel A) or M1 (0, 100, 500 or 1000 nM) (panel B) or M2 (0, 100, 500 or 1000 nM) (panel C) for 24 h. Vinclozolin, M1 or M2 were dissolved in DMSO, and all treatment groups contained 0.1% DMSO as a final concentration. Each treatment group represents the mean ± S.E.M. of four determinations from a single experiment, and these results are representative of at least three separate experiments.

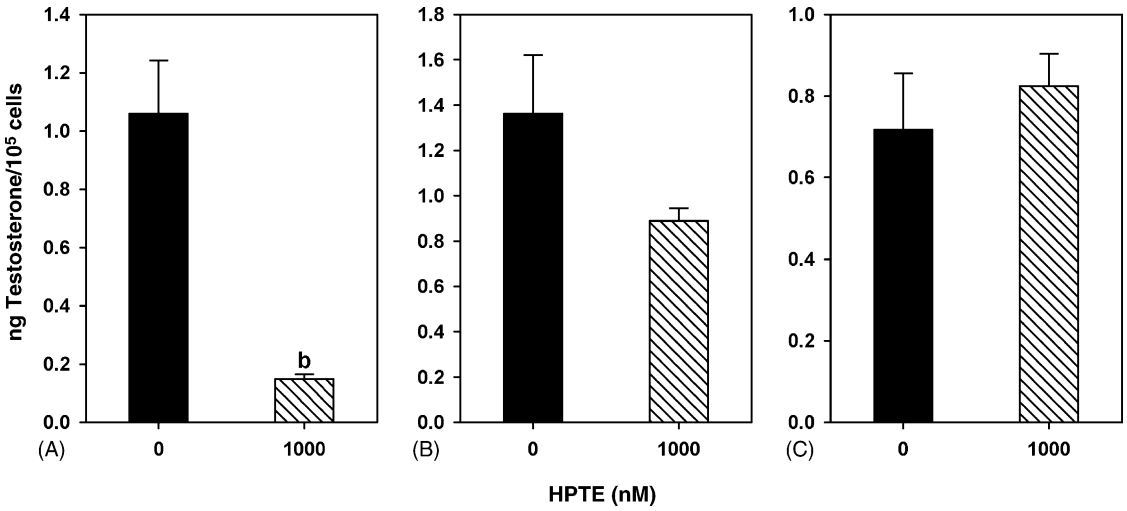

Fig. 10. Recovery of Leydig cell testosterone formation following inhibition by HPTE. Leydig cells were exposed to 10 mIU/ml hCG and 0 or 1000 nM HPTE for 4 h (panel A). Next, fresh medium containing 10 mIU/ml hCG alone was added to each well for 24 h (panel B). Following this initial recovery period, fresh medium containing 10 mIU/ml hCG was added to each well for a second 24 h recovery period (panel C). Each treatment group represents the mean ± S.E.M. of four determinations from a single experiment, and these results are representative of at least three separate experiments. (b) $P < 0.01$ when compared with the appropriate control group.

4. Discussion

The current studies have demonstrated that the reported active metabolite of MC, HPTE, progressively inhibits both basal and hCG-stimulated testosterone formation by cultured fetal Leydig cells from neonatal rats. These effects could be observed at 100 nM HPTE, and the main site of action appeared to be localized at the P450scc step which converts cholesterol to pregnenolone in the pathway of testosterone biosynthesis. MC and HPTE have been demonstrated to exhibit estrogenic or antiandrogenic properties, and their effects on sensitive cells are thought to be mediated through the ER and/or AR. However, the inability of the "pure" estrogen antagonist, ICI, to block the effects of HPTE and the lack of effects of the antiandrogenic hydroxyflutamide, vinclozolin and its reported active metabolites (M1 and M2) on androgen production suggest that neither pathway is involved in explaining HPTE effects on neonatal Leydig cells.

The observations of the current study confirm the reported decline in both basal and hCG-stimulated testosterone production in cultured Leydig cells from both immature and adult rats in response to HPTE [24]. However, in that study, the effects of HPTE were blocked by the concomitant inclusion of the pure estrogen antagonist, ICI, which suggested that HPTE acted as an estrogen, mediated through the ER. Both adult and fetal rat Leydig cells express ERα [33], so that this is a plausible explanation for the mechanism of action of HPTE. However, in the present studies the inclusion of ICI at a single high dose or progressively increasing doses did not alter the effects of HPTE. These opposing results could be explained by differences in Leydig cell age (fetal versus immature or adult) or differences in the culture conditions used. Of interest, increasing concentrations of 17β-estradiol had no effect on hCG-stimulated testosterone formation in neonatal [25] or adult [34] rat Leydig cells in previous studies, and the study by Akingbemi et al. [24] also reported a lack of effect of 17β-estradiol on testosterone production by Leydig cells.

Another potential mode of action of HPTE is that of an antiandrogen through the AR [17], which has been identified in rat Leydig cells [35]. Androgens, generally, have been reported to decrease hCG-stimulated testosterone formation in adult rat Leydig cells [36], but in immature rats, androgen was reported to stimulate Leydig cell differentiation [37]. The role, if any, of androgens in directly regulating fetal Leydig cell functional activity is not known. However, in the present studies, neither the antiandrogen, hydroxyflutamide, nor the antiandrogenic fungicide, vinclozolin (or its reported active metabolites M1 and M2), had any effect on hCG-stimulated testosterone formation by neonatal Leydig cells. These results suggest that HPTE is not acting as an antiandrogen through the AR to inhibit testosterone formation.

Why the P450scc step among the steroidogenic enzymes which convert cholesterol to testosterone is sensitive to HPTE is not clear. The study by Akingbemi et al. [24] reported that the decline in P450scc activity was due to the inhibition of mRNA for the enzyme. Although this may be one explanation for the decline, the fact that the half life for Leydig cell P450 mRNA has been estimated to be 4–8 h [38] and we observed a decrease in androgen formation by 1 h following exposure to HPTE may suggest that enzyme activity itself may be directly affected. The observation that the pattern of inhibition after exposure to HPTE is duplicated following addition of 22(*R*)-hydroxycholesterol, which readily passes through the mitochondrial membrane to gain access to P450scc localized on the inner membrane [39], would suggest that cholesterol

availability through the steroidogenic acute regulatory protein (StAR) is not involved [40]. One potential mode of action of HPTE that we explored was that its binding to P450scc caused electron leakage outside the electron transport chain to generate oxygen radicals (superoxide, peroxynitrite and/or hydroxyl radicals) [41,42], which, in turn, could initiate lipid peroxidation and damage to P450scc [43]. However, this appeared unlikely as concomitant inclusion of the water-soluble α-tocopherol derivative, Trolox (an antioxidant), did not alter the inhibition produced by HPTE. In addition, because this preparation of cells from neonatal rat testes was not 100% pure, the possibility that contaminating cells may modulate fetal Leydig cell response to HPTE should be considered.

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