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The effects of the reported active metabolite of methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, on testosterone formation by cultured Leydig cells from young adult rats

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Abstract

Methoxychlor (MC) is an insecticide that is currently used on a variety of agricultural crops, especially following the ban of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) use in the United States. Following in vivo administration, MC is converted to 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is proposed to be the active agent. Both MC and HPTE have been demonstrated to exhibit weak estrogenic and antiandrogenic activities, and they are thought to exert their effects through estrogen or androgen receptors, respectively. A recent study reported that HPTE inhibited both basal and hCG-stimulated testosterone formation by immature and adult cultured rat Leydig cells and that this effect was mediated through the estrogen receptor. In the current studies, we examined the effects of HPTE on basal and hCG-stimulated testosterone formation by cultured Leydig cells from young adult rats. In addition, we evaluated whether the effects of HPTE on rat Leydig cell testosterone biosynthesis were mediated through the estrogen receptor as an estrogen agonist or the androgen receptor as an antiandrogen. The current studies demonstrated that HPTE inhibited both basal and hCG-stimulated testosterone formation in a dose-dependent manner with significant declines in testosterone being observed at ~100 nM. The effects of HPTE were localized to the cholesterol side-chain cleavage step; however, these effects were not mediated through the classic estrogen receptor or by its acting as an antiandrogen, the currently recognized modes of action of MC and HPTE.

Keywords: Leydig cell; HPTE; Testosterone

1. Introduction

It has been hypothesized that environmental or occupational exposure to endocrine-disrupting chemicals has adversely affected the reproductive system of humans and various wildlife species [1,2]. Adverse changes in the reproductive system of males that have been reported to occur over the past 40–50 years have been attributed to gestational exposure to these chemicals [3]. These changes include an international decline in semen quality [4], and increases in the incidence of cryptorchidism [5], hypospadias [6], and testicular cancer [7]. The connection between exposure to endocrine-disrupting chemicals and the recent increase in male reproductive disorders is not universally accepted [8], and it has not been established whether these changes have any impact on male fertility [9]. Although there is controversy over the extent to which exposure to endocrine-disrupting agents has adversely affected male reproductive functions, there is general agreement for the need of additional studies to clarify the association between exposure to these agents and potential effects on human reproductive health [10].

Methoxychlor (2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane; MC) is an insecticide that is currently used on a variety of agricultural crops, especially following the ban of

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2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) use in the United States in 1972. The advantages of MC over DDT are that it is more rapidly metabolized and excreted from mammals [11], it is acutely less toxic than DDT [12] and it is biodegradable [11]. In vivo administration of MC to immature female rats increases uterine weight [13] and stimulates uterine ornithine decarboxylase activity [14], both estrogen-associated responses. In addition, following in vivo exposure, 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 [14], because HPTE binds with higher affinity to the estrogen receptor (ER) than MC [15]. However, recent studies suggest that MC itself has weak intrinsic estrogenic activity [16]. With respect to the differential activity of HPTE on the two ER isoforms (ERα and ERβ), HPTE acted as an ERα agonist in human hepatoma cells (Hep G2), but acted mainly as a competitive antagonist with ERβ [17]. In addition to estrogenic activities, MC has been reported to exhibit antiandrogenic effects following in vivo exposure to male rats [18]. In Hep G2 cells transiently transfected with the human androgen receptor (AR) and an androgen-responsive reporter, both MC and HPTE were antagonistic, although HPTE was approximately an order of magnitude more potent [19]. Thus, with respect to potential reproductive effects of MC and HPTE, the receptor sub-type (ERα or ERβ) and the type of steroid receptor (ER or AR) appear to influence the net response. With respect to antiandrogenic actions of chemicals, this could be effected by blocking AR-mediated actions (AR antagonistic action), inhibiting testosterone biosynthesis by Leydig cells and/or by inhibition of the conversion of testosterone to dihydrotestosterone (DHT) (inhibition of 5α-reductase activity) in DHT-responsive organs. Although MC and HPTE have been shown to inhibit AR-mediated transcriptional activity [19], a recent study demonstrated that HPTE can directly inhibit both basal and hCG-stimulated testosterone biosynthesis by cultured rat Leydig cells and that this response was mediated through the ER [20]. In the current studies, we examined the direct effects of HPTE on testosterone biosynthesis by cultured adult rat Leydig cells and evaluated whether these effects are mediated through the ER and/or AR.

2. Materials and methods

2.1. Animals

Young adult male Sprague–Dawley (Hla:(SD) CVF) rats (∼50 days of age) 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 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 (Type I), 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), L-ascorbic acid, dimethyl sulfoxide (DMSO), 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), 3-isobutyl-1-methylxanthine (IBMX), glutathione (reduced), cyproterone acetate and neutral alumina were purchased from Sigma Chemical Co., St Louis, MO. Bovine serum albumin (BSA, clinical reagent grade) and Ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals Inc., Costa Mesa, CA. Chloroform was from Fisher Scientific, Pittsburgh, PA. 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-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were from Life Technologies, Grand Island, NY. [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. Testosterone and 17β-estradiol were from Steraloids, Wilton, NH. Percoll was from Pharmacia, Piscataway, NJ. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, a derivative of α-tocopherol) was from Aldrich Chemical Co., Milwaukee, WI. 2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (99% pure) was from Cedra Corp., Austin, TX. Vinclozolin was from Crescent Chemical Co., Hauppage, NY. 2[[(3,5-Dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3*N*,5*N*-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) were gifts from the EPA/NHEERL, Research Triangle Park, NC, through Dr. William Kelce, Pharmacia Corp., Kalamazoo, MI. 4-Hydroxyflutamide was a gift from Schering-Plough Research Institute, Kenilworth, NJ. ICI 182,780 (ICI) was a gift from Dr. A.E. Wakeling, Zeneca Pharmaceuticals, Cheshire, England. Human chorionic gonadotropin (hCG, CR-127, 14 900 IU/mg) was a gift from NIDDK, Bethesda, MD.

2.3. Isolation and culture of Leydig cells

Animals were 57–63 days of age when sacrificed by intraperitoneal injection of pentobarbital. To obtain an adequate number of cells for each experiment, the testes from 8 to 10 animals were pooled, 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 [21]. 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 μg/ml, and they were ∼95% pure based on histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity.

Leydig cells were suspended in a 1:1 mixture of DMEM/F-12 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 [22,23]. One milliliter of cells (1×10^5 ml^{-1}) 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. Cells were exposed to various treatments for 0–24 h.

2.4. Treatment of cells

HPTE, vinclozolin, M1 and M2 were dissolved in DMSO. The final DMSO concentration in all treatment groups (including controls) was 0.1%. 22(*R*)Hydroxycholesterol, pregnenolone, progesterone, androstenedione and hydroxyflutamide were dissolved in ethanol. The final ethanol concentration in all treatment groups (including controls) was 0.1%. These concentrations of DMSO or ethanol did not affect testosterone biosynthesis or cell viability. In addition, none of the tested chemicals adversely affected cell viability based on cell morphology, attachment to culture plates or exclusion of trypan blue. Following exposure, the cell-free media were collected and stored at −20 °C until processed for testosterone quantitation.

2.5. Quantitation of testosterone

Testosterone was quantitated directly from the culture medium by radioimmunoassay (RIA) as described previously [24]. None of the chemicals tested interfered with the RIA at the concentrations used.

2.6. Measurement of P450 cholesterol side-chain cleavage activity

P450 cholesterol side-chain cleavage activity (P450scc) of cultured Leydig cells was determined by measuring the conversion of 25-[26,27-^{3}H]-hydroxycholesterol to pregnenolone and ^{3}H-labeled side-chain by utilizing a previously described procedure [25] with slight modifications. In brief, following the exposure of cultured cells to varying concentrations of HPTE for 24 h, fresh medium (0.5 ml) containing substrate (0.5 μCi, 5 μM) was added to each well, and cells were incubated for 1 h at 33 °C in a atmosphere of 95% air and 5% CO_2. Reactions were stopped by addition of 50 μl 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-labled 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 (ICN) as the scintillation fluid.

2.7. Statistical analysis

Data were 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 <0.05 was considered statistically significant. For time studies, data were analyzed using Students *t*-test.

3. Results

3.1. Effect of HPTE on basal or hCG-stimulated testosterone

In response to exposure to increasing concentrations of HPTE (1–1000 nM) alone for 4 h, progressive declines in testosterone were observed beginning at 100 nM (Fig. 1, panel A). At the highest HPTE dose, the testosterone level was 26% of control. In response to exposure to increasing HPTE concentrations for 24 h, a similar pattern of decline in testosterone formation was observed, with the androgen level falling to 11% of control at 1000 nM HPTE (Fig. 1, panel B). In response to exposure with 10 mIU/ml hCG and HPTE (1–1000 nM) for 4 h, only the two highest doses of HPTE reduced testosterone levels, with androgen levels declining to 47% of control at the highest concentration (Fig. 1, panel C). Exposure of Leydig cells to hCG and HPTE (1–1000 nM) for 24 h resulted in a progressive decline in testosterone beginning at 100 nM HPTE, with androgen levels falling to 32% of control at the highest concentration (Fig. 1, panel D). Over all, the effect of HPTE on basal testosterone formation generally was greater than its effect on hCG-stimulated testosterone. Studies also were conducted to determine whether HPTE inhibits testosterone formation prior to 4 h exposure. Significant declines in basal testosterone formation were observed at 1, 2 and 4 h (81, 53 and 39% of controls, respectively, when compared to appropriate controls) following exposure to 1000 nM HPTE (Fig. 2).

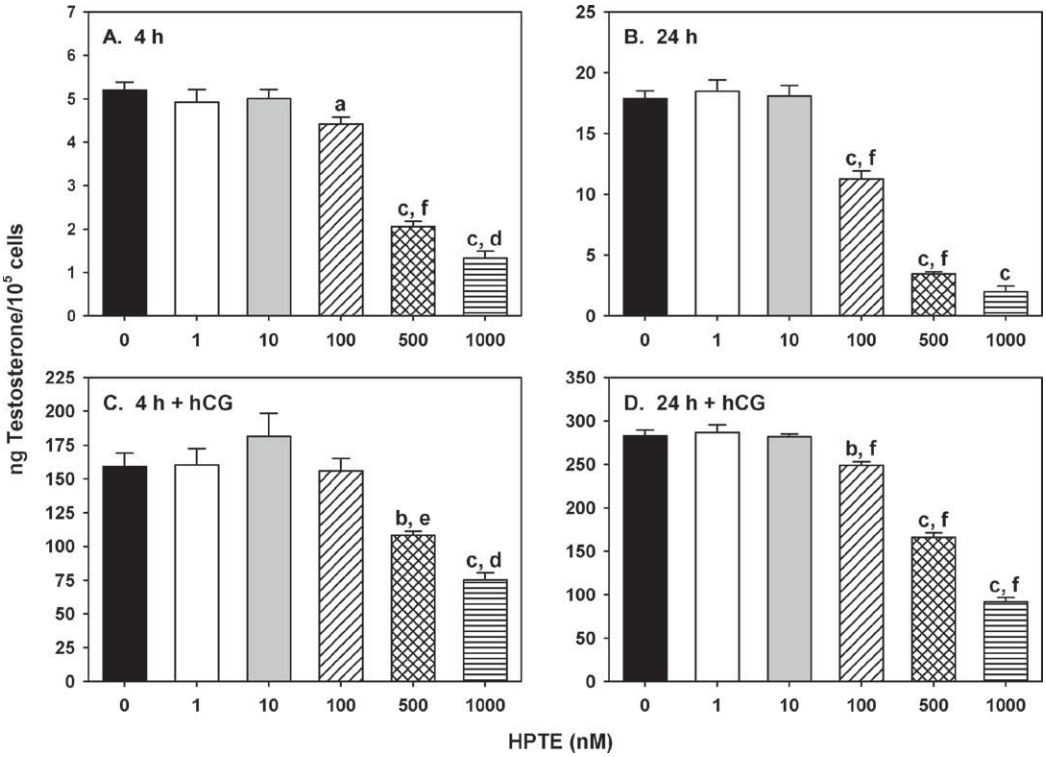

Fig. 1. Effect of HPTE on basal or hCG-stimulated testosterone. Leydig cells were exposed to HPTE alone (1–1000 nM) or HPTE (1–1000 nM) + 10 mIU/ml hCG for 4 or 24 h. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (a–c) $P < 0.05$, 0.01 and 0.001, respectively, when compared with the appropriate control group; (d–f) $P < 0.05$, 0.01 and 0.001, respectively, when compared to the appropriate immediate lower concentration.

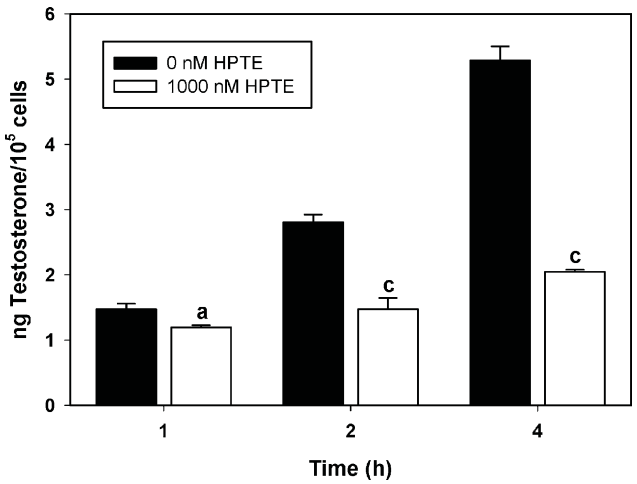

Fig. 2. Effect of time of exposure to HPTE on testosterone. Leydig cells were exposed to 1000 nM HPTE alone for 1, 2 or 4 h. Controls received vehicle alone (0.1% DMSO). Each exposed group was compared with corresponding time control. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (a) $P < 0.05$ when compared with appropriate time control; (c) $P < 0.001$ when compared to appropriate time controls.

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

Because HPTE inhibited both basal and hCG-stimulated testosterone formation by cultured Leydig cells, we evaluated whether the primary effects of HPTE occurred prior to or following the formation of cAMP. In response to exposure to 1 mM 8-Br-cAMP and HPTE (1–1000 nM) for 4 or 24 h, a progressive decline in testosterone was observed beginning at 100 nM (Fig. 3, panels A and B, respectively). At 1000 nM HPTE, testosterone levels were 50 and 29% of control following exposure for 4 or 24 h, respectively. These results suggest that the primary action of HPTE occurs following the formation of cAMP.

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

To evaluate whether exposure to HPTE inhibits a steroidogenic enzyme(s) involved in the conversion of cholesterol to testosterone, Leydig cells were exposed to HPTE (1–1000 nM) alone for 24 h. Next, fresh medium containing 10 μM 22(*R*)hydroxycholesterol, 10 μM pregnenolone, 10 μM progesterone or 10 μM androstenedione was added to each well. Following incubation for 4 h, the formation of testosterone from each of these steroid precur-

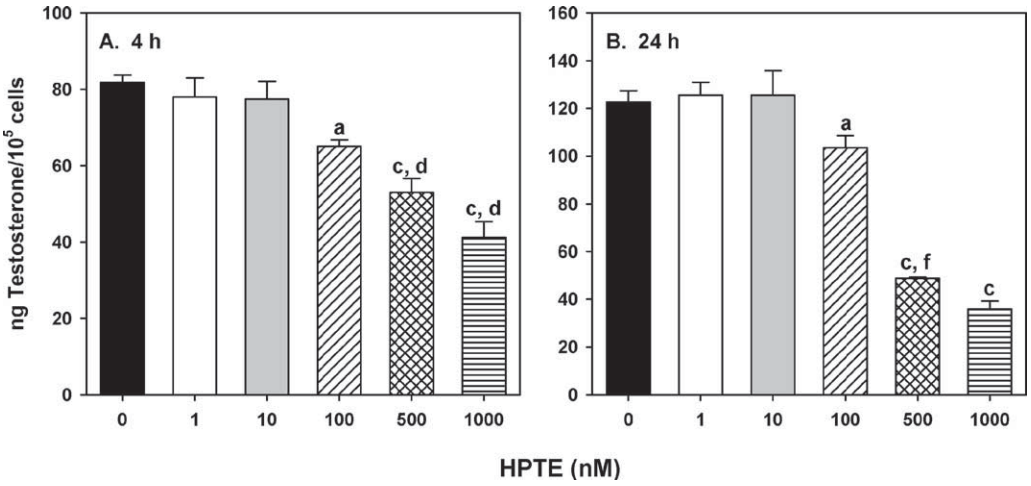

Fig. 3. Effect of HPTE on 8-Br-cAMP-stimulated testosterone. Leydig cells were exposed to HPTE (1–1000 nM) + 1 mM 8-Br-cAMP for 4 or 24 h. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (a and c) $P < 0.05$ and 0.001, respectively, when compared with the appropriate control groups; (d and f) $P < 0.05$ and 0.001, respectively, when compared to appropriate immediate lower treatment group.

sors was determined which provides an indirect measure of P450 cholesterol side-change cleavage (P450scc), 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD), P450 scc17α-hydroxylase/17–20 lyase (P450c17), and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities, respectively.

In response to the addition of 22(*R*)hydroxycholesterol, testosterone formation decreased progressively, with significant declines to 11 and 7% of control following exposure to 500 and 1000 nM HPTE, respectively (Fig. 4, panel A). In contrast, the conversion of pregnenolone (Fig. 4,

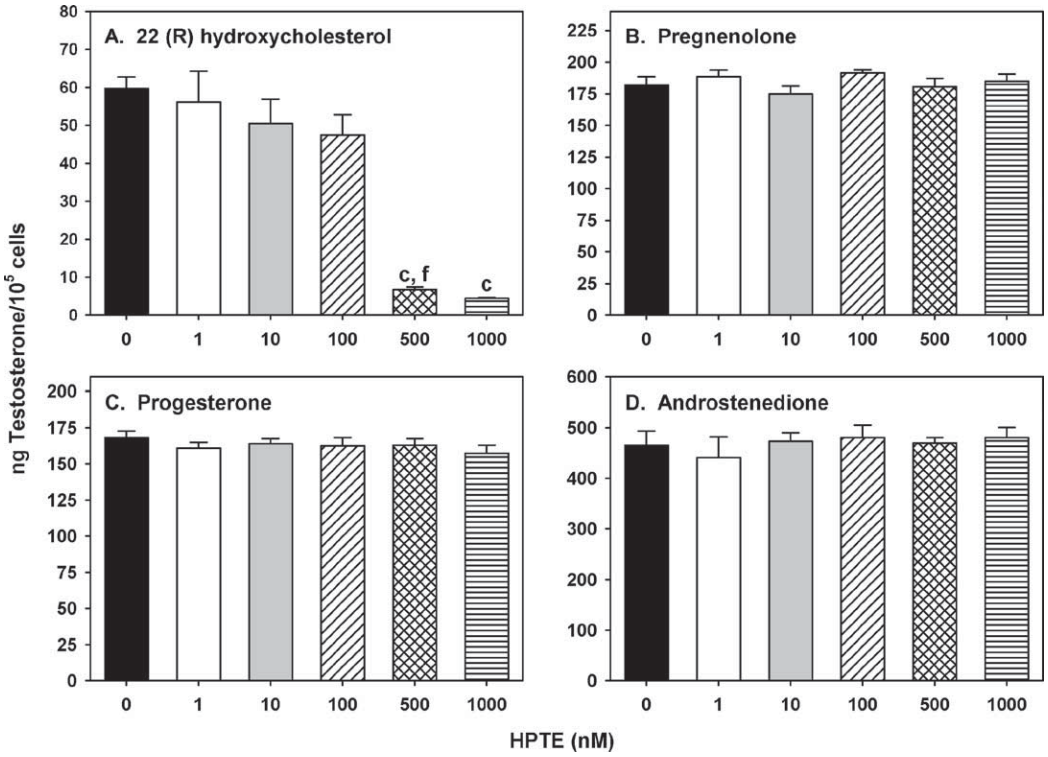

Fig. 4. Effect of initial exposure to HPTE on subsequent conversion of steroid substrates to testosterone. Leydig cells were exposed to HPTE (1–1000 nM) alone for 24 h. Next, fresh medium containing 10 μM 22(*R*)hydroxycholesterol, 10 μM pregnenolone, 10 μM progesterone or 10 μM androstenedione was added to each well, and the formation of testosterone following 4 h of incubation was determined. 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. (c) $P < 0.001$ when compared with the appropriate control; (f) $P < 0.001$ when compared to immediate lower treatment group.

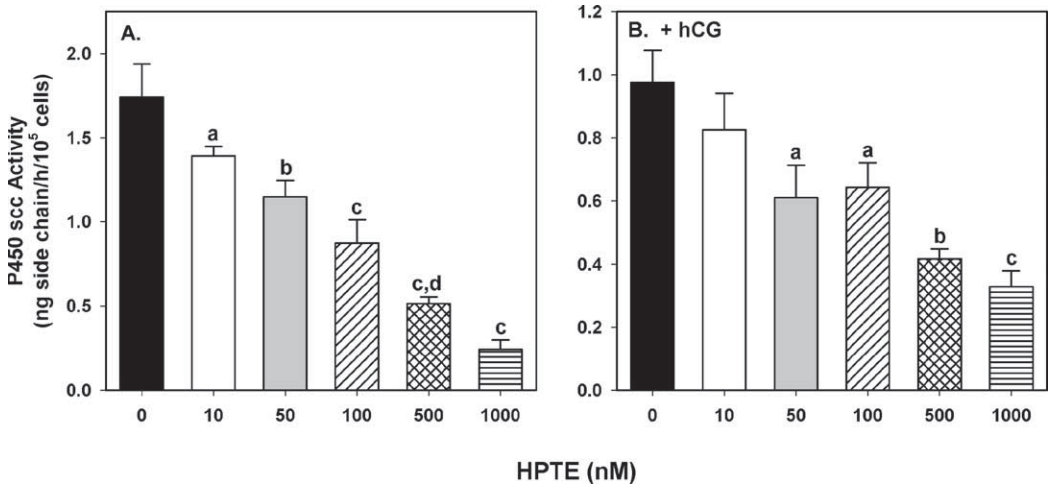

Fig. 5. Effect of HPTE on P450scc activity. Leydig cells were exposed to HPTE (10–1000 nM) without (panel A) or with 10 mIU/ml hCG (panel B) for 24 h. Following exposure, P450scc activity was estimated on intact cells. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment. These results are representative of at least three separate experiments. (a–c) $P < 0.05$, 0.01, 0.001, respectively, when compared with appropriate controls; (d) $P < 0.05$ when compared to appropriate immediate lower treatment group.

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 HPTE (1–1000 nM) and 10 mIU/ml hCG for 24 h, and similar results were observed (data not shown).

3.4. Effect of HPTE on P450scc activity

To evaluate the effect of HPTE on Leydig cell P450scc activity, Leydig cells were exposed to HPTE (10–1000 nM) alone or in the presence of 10 mIU/ml hCG or 24 h. Next, fresh medium containing 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) was added to each well. After incubation for 1 h, the release of ^{3}H-labeled side chain (localized in the aqueous phase following extraction with chloroform) was quantitated. In control cells cultured in media alone, P450scc activity was 1.74 ± 0.20 ng side-chain/h/10^5 cells (Fig. 5, panel A). Exposure to 10 nM HPTE alone for 24 h decreased activity to 1.39 ± 0.12 ng side-chain/h/10^5 cells, and exposure to higher levels of HPTE resulted in greater progressive declines in enzyme activity to 14% of control at 1000 nM. In cells exposed to increasing concentrations of HPTE and hCG for 24 h, P450scc activity of control cells was 0.98 ± 0.18 ng side-chain/h/10^5 cells (Fig. 5, panel B). Significant declines in P450scc activity was first detected at 50 nM HPTE, with

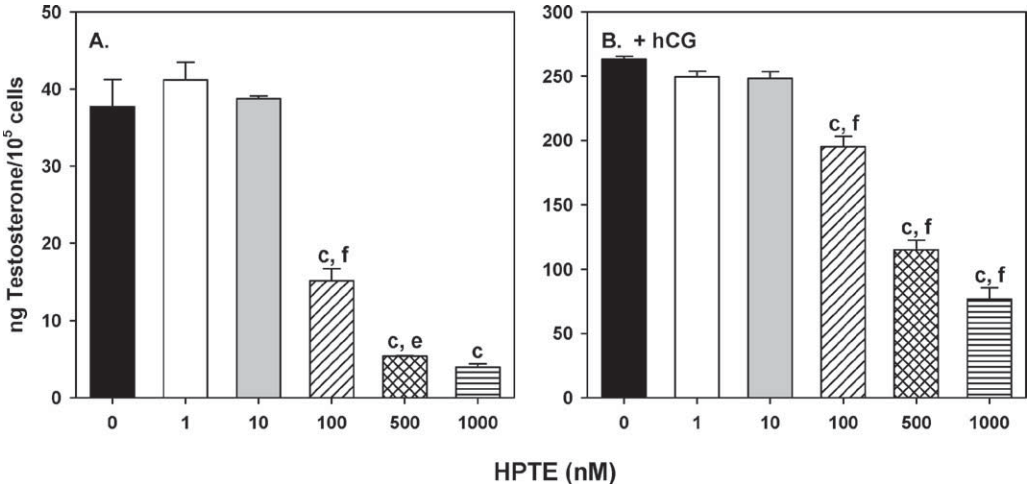

Fig. 6. Effect of concomitant exposure to ICI and HPTE on basal or hCG-stimulated testosterone. Leydig cells were exposed to 5 μM ICI and HPTE (1–1000 nM), without or with 10 mIU/ml hCG for 24 h. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments. (c) $P < 0.001$ when compared with the appropriate control; (e and f) $P < 0.01$ and 0.001, respectively, when compared with appropriate immediate lower treatment group.

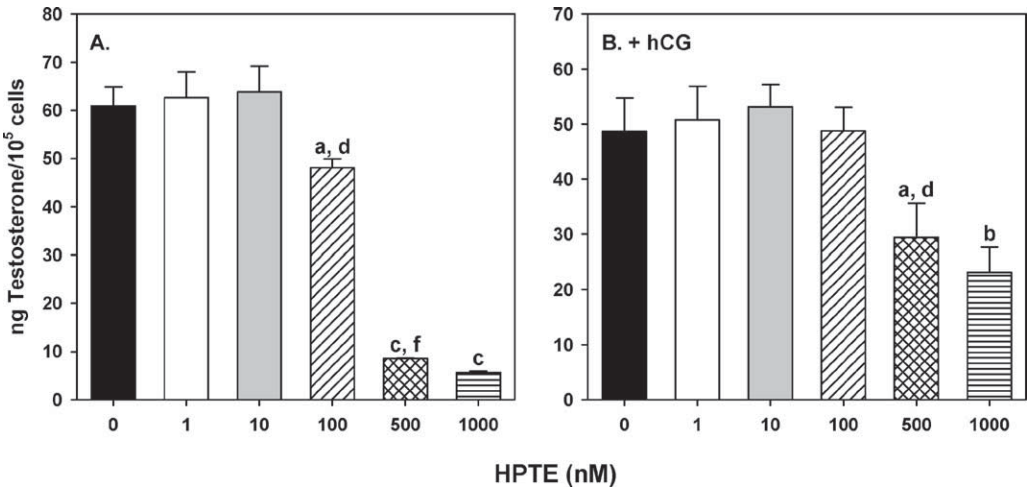

Fig. 7. Effect of concomitant exposure to ICI and HPTE, without or with hCG, on subsequent conversion of 22(*R*)hydroxycholesterol to testosterone. Leydig cells were exposed to 5 μM ICI and HPTE (1–1000 nM), without or with 10 mIU/ml hCG for 24 h. Next fresh medium containing 10 μM 22(*R*)hydroxycholesterol was added to each well. After 4 h, testosterone in the medium was quantitated. 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) $P < 0.05$, 0.01 and 0.001, respectively, when compared to appropriate controls; (d and f) $P < 0.05$ and 0.001 when compared to appropriate immediate lower treatment group.

further progressive declines in activity to 34% of control at 1000 nM HPTE.

3.5. *Effect of ICI and HPTE on testosterone formation*

To evaluate whether the effect of HPTE on testosterone formation was mediated through the ER signaling pathway, Leydig cells were exposed concomitantly to the "pure" estrogen antagonist ICI (5 μM) and HPTE (1–1000 nM) for 24 h in the absence or presence of 10 mIU/ml hCG. The inclusion of ICI with HPTE did not alter the pattern of testosterone formation, with androgen levels declining to 40, 14 and 10% of control when exposed to 100, 500 and 1000 nM HPTE and ICI (Fig. 6, panel A). Similarly, ICI did not alter the pattern of testosterone formation of cells exposed to HPTE (1–1000 nM) and 10 mIU/ml hCG for 24 h (Fig. 6, panel B). To confirm that ICI was not able to reverse the inhibitive actions of HPTE on P450scc activity, Leydig cells were exposed concomitantly to 5 μM ICI and HPTE (1–1000 nM) alone for 24 h or to 5 μM ICI, HPTE (1–1000 nM) and 10 mIU/ml hCG for 24 h. Next, fresh medium containing 22(*R*)hydroxycholesterol was added to each well, and, following incubation for 4 h, testosterone levels in the medium were determined. Under these conditions ICI did not alter

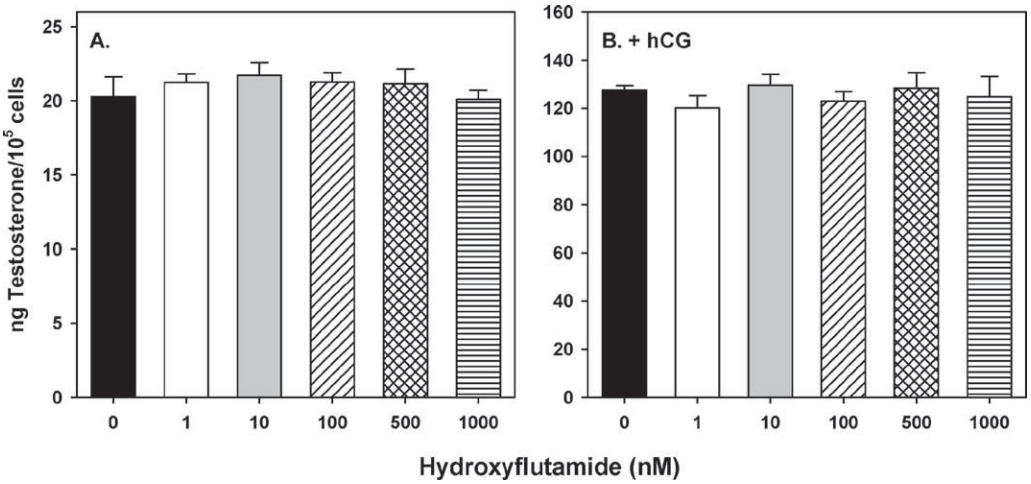

Fig. 8. Effect of hydroxyflutamide on basal or hCG-stimulated testosterone. Leydig cells were exposed to hydroxyflutamide (1–1000 nM) without or with 10 mIU/ml hCG for 24 h. Each treatment group represents the mean ± S.E.M. of four separate determinations from a single experiment, and these results are representative of at least three separate experiments.

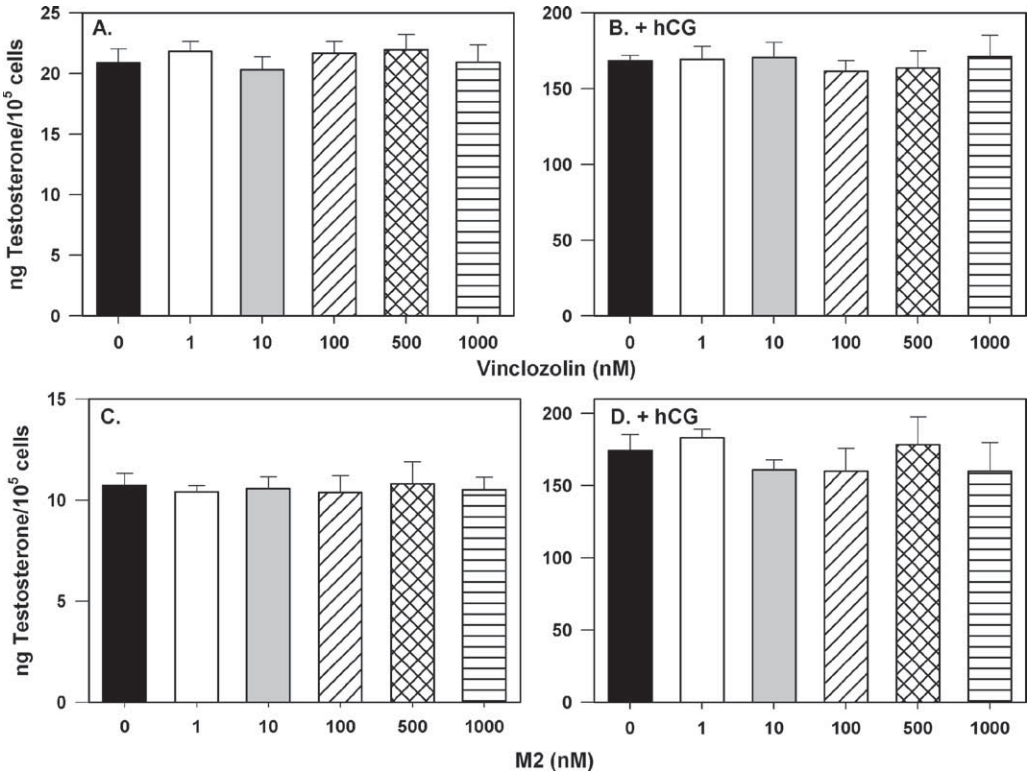

Fig. 9. Effect of vinclozolin or its active metabolite, M2, on basal or hCG-stimulated testosterone. Leydig cells were exposed to vinclozolin alone (1–1000 nM) (panel A) or vinclozolin (1–1000 nM) + 10 mIU/ml hCG (panel B), or to M2 alone (1–1000 nM) (panel C) or to M2 (1–1000 nM) + 10 mIU/ml hCG for 24 h. Both vinclozolin and M2 were dissolved in DMSO, and the final concentration of DMSO was 0.1%. Control wells received 0.1% DMSO alone. 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.

the overall pattern of 22(*R*)hydroxycholesterol conversion to testosterone (Fig. 7). These studies were repeated using lower (0.01, 0.1 and 1 μM) or higher (10 and 20 μM) ICI concentrations but the inhibition of testosterone by HPTE could not be reversed (data not shown). Furthermore, in a previous study, 17β-estradiol was without effect on testosterone biosynthesis by cultured rat Leydig cells [23].

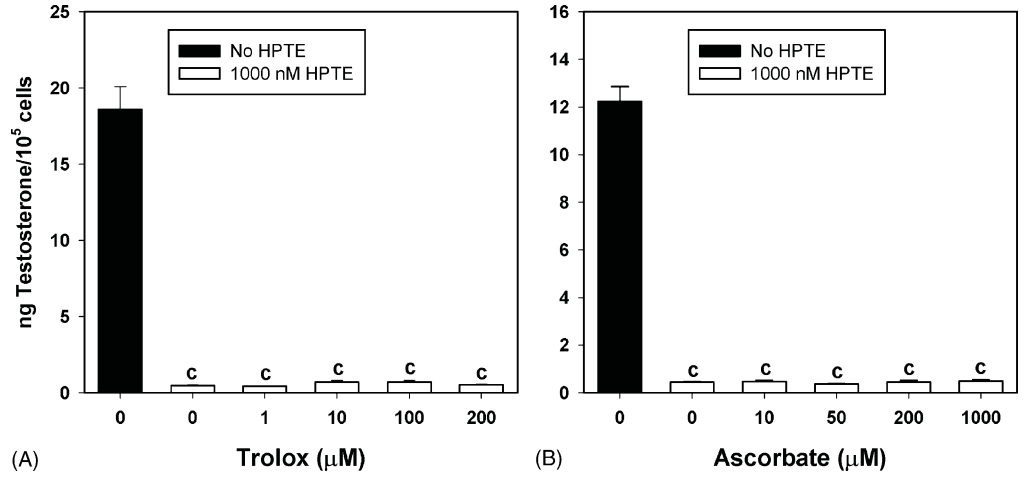

Fig. 10. Effects of trolox or ascorbate on effects of HPTE. Leydig cells were exposed to 1000 nM HPTE alone or with increasing concentrations of trolox (1–200 μM) (panel A) or ascorbate (10–1000 μM) (panel B). HPTE was dissolved in DMSO, and the final concentration of DMSO was 0.1%. Trolox was dissolved in ethanol, and the final concentration of ethanol was 0.1%. These results are the mean ± S.E.M. on four separate determinations from a single experiment, and they are representative of at least three separate experiments. (c) $P < 0.001$ when compared to respective controls receiving no HPTE or antioxidant.

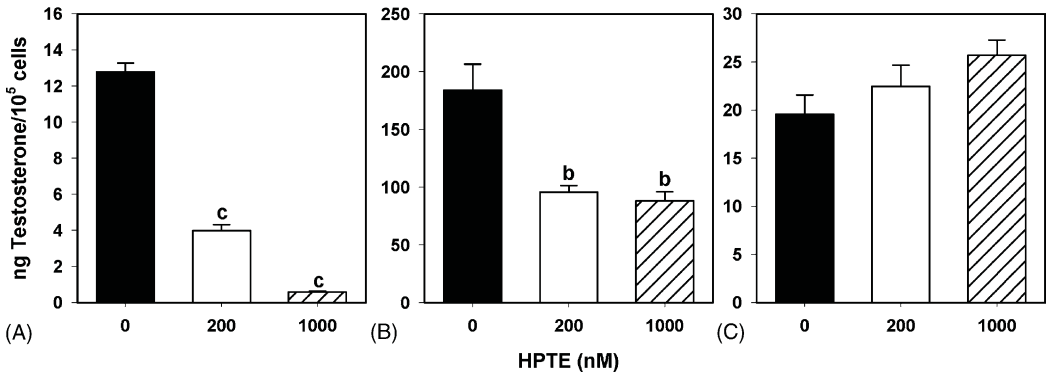

Fig. 11. Recovery from exposure to HPTE. Leydig cells were exposed to 0, 200 or 1000 nM HPTE alone for 24 h (panel A). Fresh medium containing 10 mIU/ml hCG was added to each well for the next 24 h (panel B), and fresh medium containing 10 mIU/ml hCG was added to each well for a second 24 h recovery period (panel C). Each treatment group represents the mean ± S.E.M. of four determinations from a single experiment, and they are representative of at least three separate experiments. (b and c) $P < 0.01$ and 0.001, respectively, when compared to appropriate controls.

3.6. Effects of hydroxyflutamide, vinclozolin or its active metabolite on basal or hCG-stimulated testosterone

To evaluate whether the effects of HPTE on androgen formation were due to its antiandrogenic properties, Leydig cells were exposed to the antiandrogen, hydroxyflutamide (1–1000 nM), alone or with 10 mIU/ml hCG, for 24 h. Following exposure, the level of testosterone released in the medium was quantitated. Hydroxyflutamide had no effect on either basal (Fig. 8, panel A) or hCG-stimulated (Fig. 8, panel B) testosterone levels. These studies were repeated using a second antiandrogen, cyproterone acetate. Similarly, cyproterone acetate had no effect on either basal or hCG-stimulated testosterone following exposure for 24 h (data not shown).

Vinclozolin is a fungicide currently used in both the United States and Europe. Its active metabolites are reported to be 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methyl-but-3-enanilide (M2) [26]. The parent compound, M1 and M2 are reported to exhibit antiandrogenic activity (order of potency: M2 > M1 > vinclozolin) [27]. Because HPTE is reported to have antiandrogenic activity in previous studies, whether vinclozolin or M2 (the more potent vinclozolin metabolite) had similar effects as HPTE on Leydig cell steroidogenesis was evaluated. Treatment of Leydig cells with vinclozolin (1–1000 nM), or M2 (1–1000 nM), either alone or with 10 mIU/ml hCG, had no effect on testosterone formation following exposure for 24 h (Fig. 9). Studies with M1 yielded similar results (data not shown). These studies suggest collectively that the effects of HPTE on Leydig cell androgen formation are not due to its antiandrogenic properties.

3.7. Effects of trolox or ascorbate on the effects of HPTE on Leydig cell testosterone formation

To evaluate whether the decline in testosterone formation following exposure to HPTE was the result free radical generation, which caused lipid peroxidation and damage to P450scc activity, Leydig cells were exposed concomitantly to the lipid-soluble antioxidant trolox (1–200 μM) (Fig. 10, panel A) or the water-soluble antioxidant ascorbate (1–1000 μM) (Fig. 10, panel B) and 1000 nM HPTE alone for 24 h. In the presence of 1000 nM HPTE alone, testosterone levels declined to less than 5% of control. Neither of the antioxidants examined were able to reverse the inhibitive effects of HPTE on testosterone (Fig. 10). In preliminary studies, we examined whether these concentrations of trolox or ascorbate alone had any effect on testosterone formation over 24 h, and observed that they had no effects on androgen levels in Leydig cells (data not shown).

3.8. Recovery of Leydig cell steroidogenic capacity following exposure to HPTE

To evaluate whether Leydig cells recover their capacity to synthesize testosterone following exposure to HPTE, Leydig cells were exposed to 0, 200 or 1000 nM HPTE alone for 24 h. Following exposure, 1 ml fresh medium containing 10 mIU hCG without HPTE was added to each well. After 24 h the medium was collected from each well, and this treatment was repeated for a second 24 h recovery period. In response to 200 and 1000 nM HPTE, testosterone levels declined from 12.8 ± 0.5 ng/10^5 cells in control cells to 4.0 ± 0.3 (31% of control) and 0.6 ± 0.04 ng/10^5 cells (5% of control), respectively (Fig. 11, panel A). Removal of HPTE and the inclusion of 10 mIU/ml hCG to all wells increased testosterone levels in the control group to 183.8 ± 22.6 ng/10^5 cells and to 95.6 ± 6.0 (52% of control) and 88.0 ± 8.1 ng/10^5 cells (48% of control) in cells initially exposed to 200 and 1000 nM HPTE, respectively (Fig. 11, panel B). These values represent only a partial recovery of their capacity to synthesize testosterone. The addition of fresh medium and hCG for a second 24 h recovery period resulted in testosterone levels of 19.6 ± 2.0 ng/10^5 cells in control Leydig cells, while testosterone levels in cells initially exposed to 200 or 1000 nM HPTE were

22.5 ± 2.2 (115% of control) and 25.7 ± 1.6 ng/10^5 cells (131% of control), respectively (Fig. 11, panel C). These values were not significantly different from the corresponding control value. The decline in testosterone levels on day 3 of culture in control cells represents the reported decline in the capacity of adult rat Leydig cells to synthesize this androgen over days 1–3 in culture [28]. The inclusion of hCG during the 2-day recovery period following exposure to HPTE was necessary to observe recovery of androgen levels. In the absence of hCG, testosterone levels did not return to control levels (data not shown).

4. Discussion

The studies described herein have demonstrated that HPTE (the reported active metabolite of MC) progressively inhibits both basal and hCG-stimulated testosterone formation by cultured Leydig cells from young adult rats. The effects of HPTE were observed at concentrations of 100 nM and lower. The primary locus of action of HPTE appeared to be the P450scc step, which converts cholesterol to pregnenolone. Although the actions of MC and HPTE are generally ascribed to their weak estrogenic or antiandrogenic properties, mediated through the ER or AR, respectively, the inability of the "pure" estrogen antagonist, ICI, to alter the effects of HPTE and the lack of effects of the antiandrogens, hydroxyflutamide, vinclozolin and its active metabolite, M2, to alter androgen formation in Leydig cells, suggest that neither pathway/mechanism is involved.

The current observations confirm the results of a previous study with respect to a decline in basal and hCG-stimulated testosterone formation by cultured adult rat Leydig cells following exposure to HPTE [20]. However, in that study, the inhibitive effects of HPTE were reversed by concomitant exposure to ICI, thereby suggesting that the effects of HPTE were mediated through the ER pathway. In the present studies, ICI did not alter the effects of HPTE. It has been demonstrated previously that rat Leydig cells express mainly ERα [29]. Although we do not have an explanation for these differences, they may, in part, be associated with differences in media composition, cell density or the age of animals from which Leydig cells were isolated. It should be noted that we did not observe any effects of 17β-estradiol at doses of 1–1000 nM on basal or hCG-stimulated testosterone formation of cultured Leydig cells from young adult rats in a previous study [23], and the study by Akingbemi et al. [20], similarly, reported no effects of 17β-estradiol on Leydig cell testosterone formation.

Another potential mechanism of action of HPTE is that of an antiandrogen through the AR [19], and ARs have been identified in rat Leydig cells [30]. However, androgens, generally, have been reported to inhibit hCG-stimulated testosterone formation by rat Leydig cells, and cyproterone acetate was able to reverse the inhibitive effects of the synthetic androgen, R 1881 [31]. In cultured mouse Leydig cells, testosterone was reported to inhibit the activity and amount of cytochrome P450c17, which converts progesterone to androstenedione [32] and this, potentially, could regulate the net levels of testosterone produced by Leydig cells. However, in immature rats, androgen was reported to enhance the differentiation of immature Leydig cells [33]. In the current studies, neither hydroxyflutamide or the antiandrogenic fungicide, vinclozolin, or its reported active metabolite, M2, had any effect on basal or hCG-stimulated testosterone formation, suggesting that the antiandrogenic properties of HPTE are not associated with the inhibition of testosterone formation by rat Leydig cells.

Why HPTE specifically targets the P450scc step in the steroidogenic pathway of androgen biosynthesis is not known, although a previous study also identified this as the sensitive step [20]. We examined the possibility that HPTE acted as a pseudosubstrate to bind to the active site of P450scc. Because HPTE is not coupled to the electron transport chain that transfers electrons from NADPH for substrate hydroxylation(s), potentially, this could cause excessive electron leakage outside the electron transport chain to generate reactive oxygen species (superoxide, peroxynitrite and/or hydroxyl radicals) [34,35], and these oxygen radicals could initiate lipid peroxidation and damage to P450scc [36]. However, in the current studies, concomitant exposure to increasing concentrations of two different antioxidants did not reverse the inhibitive effects of HPTE, suggesting that the adverse effect on Leydig cell testosterone formation is not mediated by the generation of reactive oxygen species, which might cause damage to P450scc.

In comparing the current in vitro results with previous in vivo studies, daily exposure of Long-Evans hooded male rats by gavage from the day of weaning to about days 97–100 with 100 or 200 mg MC/kg/day generally reduced circulating testosterone levels, hCG-stimulated testosterone formation by decapsulated intact testes and caudal epididymal sperm count without adversely affecting fertility [37]; however, daily exposure of Long-Evans hooded male rats from weaning to about 11 months of age did not produce significant declines in circulating testosterone levels [18], suggesting that these animals become less sensitive to MC as they age. In another study, pregnant rats were dosed daily from gestation day 14 to postnatal day 7, followed by daily direct dosing of the pups from postnatal days 7 to 42 with 5, 50 or 150 mg MC/kg/day. This treatment caused minimal changes in testes, although the number of spermatids per testis was reduced at the 50 mg/kg dose [38]. It cannot be ascertained whether this change was associated with a decline in testicular and/or circulating testosterone levels because androgen levels were not reported. Thus, in general, in vivo exposure of male rats to MC appears to have less effect on Leydig cell testosterone formation than following direct exposure of Leydig cells. Nevertheless, our current results demonstrate that the two current prevailing concepts on the mode of action of MC and HPTE (acting as a weak estrogen through

the ER or as an antiandrogen through the AR), do not apply with respect to Leydig cell steroidogenesis. Whether a similar pattern can be demonstrated in other reproductive tissues remains to be determined.

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References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;101:378–84.
[2] Neubert D. Vulnerability of the endocrine system to xenobiotic influence. Regul Toxicol Pharmacol 1997;263:9–29.
[3] Sharpe RM, Skakkebaek NE. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993;341:1392–5.
[4] Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. Br Med J 1992;305:609–13.
[5] John Ratcliffe Hospital Cryptorchidism Study Group. Cryptorchidism. A prospective study of 7500 consecutive male births, 1985–1988. Arch Dis Childhood 1992;67:892–903.
[6] Paulozzi LJ, Erickson JD, Jackson RJ. Hypospadias trends in two US surveillance systems. Pediatrics 1997;100:831–4.
[7] Brown LM, Pottern LM, Hoover RN, Devesa SS, Aseiton P, Flannery JT. Testicular cancer trends in the United States: trends in incidence and mortality. Int J Epidemiol 1986;15:164–70.
[8] Safe SH. Environmental and dietary estrogens and health: is there a problem? Environ Health Perspect 1995;103:346–51.
[9] Lamb DJ. Hormonal disruptors and male infertility: are men at serious risk? Regul Toxicol Pharmacol 1997;26:30–3.
[10] Cooper RL, Kavlock RJ. Endocrine disruptors and reproductive development: a weight-of-evidence overview. J Endocrinol 1997;152:159–66.
[11] Kapoor JP, Metcalf RL, Nystrom RF, Sangha GK. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects and in a model ecosystem. J Agric Food Chem 1970;18:1145–52.
[12] Hayes WJ. Clinical handbook on economic poisons. Chamblee, GA: Environmental Protection Agency Pesticides Program; 1971.
[13] Welch RM, Levin W, Conney AH. Estrogenic action of DDT and its analogs. Toxicol Appl Pharmacol 1969;14:358–67.
[14] Bulger WH, Muccitelli RM, Kupfer D. Studies on the in vivo and in vitro estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxygenase in methoxychlor activation. Biochem Pharmacol 1978;27:2417–23.
[15] Bulger WH, Muccitelli RM, Kupfer D. Interactions of methoxychlor, methoxychlor base-soluble contaminant, and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane with uterine estrogen receptor. J Toxicol Environ Health 1978;4:881–93.
[16] Elsby R, Maggs JL, Ashby J, Paton D, Sumpter JP, Park BK. Assessment of the effects of metabolism on the estrogenic activity of xenoestrogens: a two-stage approach coupling human liver microsomes and a yeast estrogenicity assay. J Pharmacol Exp Ther 2001;296:329–37.
[17] Gaido KW, Leonard LS, Maness SC, Hall JM, McDonnell DP, Saville B, et al. Differentical interaction of methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β. Endocrinology 1999;140:5746–53.
[18] Gray LE, Ostby J, Cooper RL, Kelce WR. The estrogenic and antiandrogenic pesticide methoxychlor alters the reproductive tract and behavior without affecting pituitary size and prolactin secretion in male rats. Toxicol Ind Health 1999;15:37–47.
[19] Maness SC, McDonnell DP, Gaido KW. Inhibition of androgen receptor-dependent transcriptional activity by DDT and methoxychlor in HepG2 human hepatoma cells. Toxicol Appl Pharmacol 1998;151:135–42.
[20] Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat Leydig cells through suppression of steady-state messenger ribonucleic acid levels of cholesterol side-chain cleavage enzyme. Biol Reprod 2000;62:571–8.
[21] Klinefelter GR, Hall PF, Ewing LL. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. Biol Reprod 1987;36:769–83.
[22] Murono EP, Derk RC, de León JH. Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats. Reprod Toxicol 1999;13:451–62.
[23] Murono EP, Derk RC, de León JH. Differential effects of octylphenol, 17β-estradiol, endosulfan, or bisphenol A on the steroidogenic competence of cultured adult rat Leydig cells. Reprod Toxicol 2001;15:551–60.
[24] Murono EP, Lin T, Osterman J, Nankin HR. The effects of cytochalasin B on testosterone synthesis by interstitial cells of rat testis. Biochem Biophys Acta 1989;633:228–36.
[25] Georgiou M, Perkins LM, Payne AH. Steroid synthesis-dependent, oxygen-mediated damage of mitochondrial and microsomal P-450 enzymes in rat Leydig cell cultures. Endocrinology 1987;121:1390–9.
[26] Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LR. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicol Appl Pharmacol 1994;126:276–85.
[27] Wong C, Kelce WR, Sar M, Wilson EM. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J Biol Chem 1995;270:19998–20003.
[28] Klinefelter GR, Ewing LL. Maintenance of testosterone production by purified adult rat Leydig cells for 3 days in vitro. In Vitro Cell Dev Biol 1989;25:283–8.
[29] Fisher JS, Miller MM, Majdic G, Saunders PTK, Fraser HM, Sharpe RM. Immunolocalization of oestrogen receptor-α (ERα) within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. J Endocrinol 1997;153:485–95.
[30] Gulizia S, D'Agata R, Sanborn BM, Steinberger E. Evidence for the presence of androgen receptors in purified rat Leydig cells. J Androl 1983;4:248–52.
[31] Adashi EY, Hseuh AJW. Autoregulation of androgen production in a primary culture of rat testicular cells. Nature 1981;193:737–8.
[32] Hales DB, Sha L, Payne AH. Testosterone inhibits cAMP-induced de novo synthesis of Leydig cell cytochrome P-45017α by an androgen receptor-mediated mechanism. J Biol Chem 1987;262:11200–6.
[33] Shan L-X, Hardy DO, Catterall JF, Hardy MP. Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors in vivo. Endocrinology 1995;136:1686–93.
[34] Hornsby PJ, Crivello JF. The role of lipid peroxidation and biological oxidations in the function of the adrenal cortex. Mol Cell Endocrinol 1983;30:1–20.
[35] Hanukoglu I, Rapoport R, Weiner L, Sklan D. Electron leakage from the mitochondrial NADPH-adrenodoxin reductase-adrenodoxin-P450scc (cholesterol side chain cleavage) system. Arch Biochem Biophys 1993;305:489–98.

[36] Hornsby PJ. Regulation of cytochrome P-450-supported 11β-hydroxylation of deoxycortisol by steroids, oxygen and antioxidants in adrenocortical cell cultures. J Biol Chem 1980;255:4020–7.

[37] Gray LE, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, et al. A dose–response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. Fundam Appl Toxicol 1989;12:92–108.

[38] Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, et al. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. Fundam Appl Toxicol 1997;40:138–57.