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The methoxychlor metabolite, HPTE, directly inhibits the catalytic activity of cholesterol side-chain cleavage (P450scc) in cultured rat ovarian cells☆

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Abstract

Exposure to the pesticide methoxychlor in rodents is linked to impaired steroid production, ovarian atrophy and reduced fertility. Following *in vivo* administration, it is rapidly converted by the liver to 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), the reported active metabolite. Both methoxychlor and HPTE have weak estrogenic and antiandrogenic activities, and these effects are thought to be mediated through the estrogen and androgen receptors, respectively. Previous *in vivo* studies on methoxychlor exposure to female animals have demonstrated decreased progesterone production but no change in serum estrogen levels. We recently showed that HPTE specifically inhibits the P450 cholesterol side-chain cleavage (P450scc, CYP11A1) step resulting in decreased androgen production by cultured rat testicular Leydig cells. The current studies examined the mechanism of action of HPTE on progesterone production by cultured ovarian cells (granulosa and theca-interstitial) from pregnant mare serum gonadotropin-primed immature rats. In addition, we evaluated whether the effects of HPTE on rat ovarian cell progesterone biosynthesis were mediated through the estrogen or androgen receptors. Exposure to HPTE (0, 10, 50 or 100 nM) alone progressively inhibited progesterone formation in cultured theca-interstitial and granulosa cells and the P450scc catalytic activity in theca-interstitial cells in a dose-dependent manner with significant declines starting at 50 nM. However, HPTE did not change mRNA levels of the P450scc system (P450scc, adrenodoxin reductase and adrenodoxin) as well as P450scc protein levels. Of interest, estradiol, xenoestrogens (bisphenol-A or 4-*tert*-octylphenol), a pure antiestrogen (ICI 182,780), or antiandrogens (4-hydroxyflutamide or the vinclozolin metabolite M-2), had no effect on progesterone production even at 1000 nM. Co-treatment of HPTE with ICI 182,780 did not block the effect of HPTE on progesterone formation. These studies suggest that the decline in progesterone formation following exposure to HPTE in cultured ovarian cells is associated with the inhibition of catalytic activity of P450scc at least in theca-interstitial cells. This action does not appear to be mediated through the estrogen or androgen receptor signaling pathways, and other chemicals exhibiting estrogenic, antiestrogenic or antiandrogenic properties do not mimic its effect on ovarian steroid production.
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1. Introduction

Methoxychlor (MC) is an insecticide that is released into the environment mainly as a result of its application to agricultural crops, livestock, animal feed, home gardens and pets [1]. *In vivo* exposure to MC in laboratory animals has been reported to cause embryonic toxicity [2], as well as precocious puberty [3], decreased fertility [4], ovarian atrophy [5–7] and a dose-dependent decline in the number of follicles [8] and ovarian steroid production [9]. Following *in vivo* administration of MC, it is rapidly converted to demethylated and hydroxylated metabolites by the liver cytochrome P450 (CYP) enzymes [10,11]. In addition to the liver, there is evidence for peripheral metabolism of MC. For example, the ovarian surface epithelium expresses CYP enzymes that are capable of metabolizing MC [12]. It is generally accepted that the physiological effects of *in vivo* MC treatment are mediated mainly by the active metabolite, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)

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[13]. In female rodents, exposure to MC and HPTE caused a uterotropic response and the proliferation of ovarian surface epithelium through estrogen receptor (ER)-linked mechanisms [14–16]. Both MC and HPTE have weak estrogenic and antiandrogenic activities [17–19]. However, HPTE binds with higher affinity to the ER [13] and is a more potent androgen receptor (AR) blocker [19]. In human hepatoma cells (HepG2), HPTE acted as an ERα agonist; however, it was antagonistic to ERβ and AR [20]. Although it is generally thought that chemicals such as HPTE exert their effects through receptor-mediated pathways, a recent study suggested that some actions of HPTE on steroidogenesis may not be mediated through the ER or AR [21]. For example, Murono et al. demonstrated that the concomitant addition of the "pure" antiestrogen, ICI, did not block the inhibition of testosterone formation by HPTE, and the exposure to the antiandrogenic hydroxyflutamide or vinclozolin did not mimic the effect of HPTE on androgen production by cultured fetal Leydig cells from neonatal male rats [21].

Regarding the effects of MC on gonadal steroidogenesis, previous *in vivo* studies in female and male rodents demonstrated decreased serum progesterone and androgen levels, respectively, following exposure to MC [4,9,22], and the effects of HPTE on testis steroidogenesis localized to the P450 cholesterol side-chain cleavage (P450scc) step in the pathway of androgen production from cholesterol [21–24]. It has not yet been established how HPTE alters ovarian steroid production. In the current studies, we examined the possible mechanism(s) of action of HPTE on progesterone production by cultured ovarian cells from pregnant mare serum gonadotropin (PMSG)-primed immature rats. In addition, we evaluated whether the effects of HPTE on rat ovarian cell progesterone biosynthesis were mediated through the ER or AR.

2. Materials and methods

2.1. Animals

Immature (17-day-old) female Sprague–Dawley rats (certified virus free, 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) until the age of 21 days when they were weaned. At this age, three to four pups were placed in a polycarbonate shoebox cage containing a mixture of Alpha-dri (Shephard Specialty Paper, Watertown, TN) and Beta Chip (virgin hardwood chips from NEPCO, Warrenburg, NY) as the bedding material. They were exposed to a 12 h light and 12 h dark cycle, and they received Teklad 2918 rat chow (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 NIOSH Animal Care and Use Committee.

2.2. Reagents

Collagenase (Sigma Blend Type L), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNase I), dimethylsulfoxide (DMSO), pregnant mare serum gonadotropin (PMSG) and neutral alumina were purchased from Sigma, St. Louis, MO, USA. Ecolite (liquid scintillation fluid) was 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), phosphate buffered saline (PBS) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY, USA. 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. 2,2,Bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, 99% pure) was from Cedra Corp., Austin, TX, USA. 17β-Estradiol (estradiol) was from Steraloids, Wilton, NH, USA. 4,4′-Isopropylidenediphenol (bisphenol A) and 4-*tert*-octylphenol (octylphenol) were from Aldrich Chem. Co., Milwaukee, NY. 3′,5′-Dichloro-2-hydroxy-2-methylbut-3-enanilide (M-2, a metabolite of the fungicide vinclozolin with antiandrogenic properties) was a gift from the EPA/NHEERL, Research Triangle Park, NC, USA, through Dr. William Kelce, Pharmacia Corp., Kalamazoo, MI, USA. 4-Hydroxyflutamide (flutamide) was a gift from Schering-Plough Research Corp., Kenilworth, NJ, USA. ICI 182,780 (ICI) was a gift from Dr. A.E. Wakeling, Zeneca Pharmaceuticals, Cheshire, UK.

2.3. Treatment of animals

Animals were 23–25 days of age at the start of treatment. Each animal received a single injection (s.c.) of PMSG dissolved in PBS (20 IU/0.1 ml). Animals were treated between 08:00 and 10:00 h each day. Approximately 48 h following exposure to PMSG, animals were sacrificed by intra-peritoneal pentobarbital injection (∼200 mg/kg). For each experiment, three to four animals were sacrificed to yield a single pool of cells for culture in a given study.

2.4. Isolation and culture of theca-interstitial and granulosa cells

Granulosa cells (GC) and theca-interstitial (TI) cells were isolated by slight modifications of methods described previously [25,26]. Briefly, the ovaries (three to four pairs) were harvested, stripped of bursa and fat tissue and then punctured several times with a 26-gauge needle until all the antral follicles were ruptured to release GC. The medium containing GC was centrifuged (209 × *g*) for 10 min to yield GC. The final GC pellet was then resuspended in DMEM/F-12 medium. The residual ovarian tissue was washed twice with fresh medium and incubated in a rotary shaker bath for 10 min (37 °C) in 10 ml of collagenase–DNase-1 solution (0.75 mg/ml of collagenase, 1 μg/ml of DNase-1 in medium 199). The dissociated cells were discarded to remove the residual GC, and the remaining tissue was further digested with collagenase for an additional 50 min. Finally, ice-cold medium 199 was added to stop further dissociation of the residual tissue, and the cell suspension was centrifuged (209 × *g*) for 10 min to yield TI cells. The final pellet was then resuspended in DMEM/F-12 medium.

GC and TI cells (10^5/well) were plated into 24-well Costar culture plates in 1 ml of a 1:1 mixture of DMEM/F-12 medium containing 15 mM HEPES (pH 7.4), 15 mM $NaHCO_3$, 100 U/ml penicillin G and 100 μg/ml streptomycin but lacking phenol red as described previously [25]. Cells were exposed to concentrations of 0, 10, 50 or 100 nM HPTE on the day of plating and cultured for 24 h in DMEM/F12 medium at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The HPTE concentrations were chosen based on preliminary studies conducted to determine the sensitivity of cultured GC and TI to the chemical. Furthermore, cultured GC and TI cells became detached from culture plates at HPTE concentrations above 1 μM. In addition, although plated cells survived more than 7 days under the culture conditions used, their steroid output declined after about 24 h.

2.5. Measurement of progesterone

Progesterone was quantitated directly from the collected medium using a radioimmunassay (RIA) kit as described in the manufacturer's protocol (Diagnostic Products Corporation, Los Angeles, CA).

2.6. Measurement of P450 cholesterol side-chain cleavage activity

P450scc activity of intact TI cells and GC 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 [27] with slight modifications [23]. In brief, isolated TI cells (2×10^5/0.5 ml medium) or GC (5×10^5/0.5 ml medium) were plated into 24-well culture plates. Varying concentrations of HPTE (0, 10, 50, 100 or 500 nM) were added at the time of plating, and after exposure for 8 h, 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM)

was added to each well. The cells were incubated for an additional 16 h at 37 °C in an atmosphere of 95% air and 5% CO_2. Reactions were stopped by adding 50 μl of 1 N NaOH to each well. The contents of each well were transferred to a 7 ml borosilicate glass vial, and each well was washed with 1.05 ml PBS. The PBS washes were transferred to the vials containing the corresponding incubation medium, and the contents of each vial 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). The aqueous phase and the neutral alumina were mixed, and the tubes were centrifuged (∼1640 × *g*) for 20 min to settle the neutral alumina. An aliquot of the aqueous phase was removed and counted using ecolite as the scintillation fluid.

2.7. Quantitation of mRNAs by real-time RT-PCR (reverse-transcription polymerase chain reaction)

The mRNA levels were measured using primer sets designed by Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) and synthesized by Operon (Huntsville, AL) and performed by the ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Total RNA was isolated using RNAqueous-4PCR kits (Ambion, Austin, TX) from TI cells (∼3 million) cultured in the absence or presence of 100 nM HPTE. The DNAse I-treated RNA (0.5–1 μg) was reverse transcribed, using Superscript II (Life Invitrogen, Gaithersburg, MD). The cDNA generated was diluted 1:100, and 7.25 μl was used to conduct the PCR reaction according to the real-time PCR kit instructions. The comparative C_T (threshold cycle) method was used to calculate the relative concentrations (User Bulletin 2, ABI PRISM 7700 sequence detector, PE Applied Biosystems, Foster City, CA). Briefly, the method involves obtaining the C_T values for the genes of P40scc (CYP11A1), steroidogenic acute regulatory protein (StAR), adrenodoxin (ADN) and adrenodoxin reductase (ADR), normalizing to a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and deriving the fold increase compared to the control group (Table 1).

2.8. Western blot analysis

TI cells (2×10^6/dish) were plated into 100 mm Petri dishes in 10 ml of a 1:1 mixture of DMEM/F-12 medium containing 15 mM HEPES (pH 7.4), 15 mM $NaHCO_3$, 100 U/ml penicillin G, and 100 μg/ml streptomycin as described previously [25]. Cells were exposed to concentrations of 0, 20 or 100 nM HPTE on the day of plating and cultured for 24 h in DMEM/F12 medium at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cultured cells were then scraped off the dishes using a cell scraper, and the cells were resuspended in 20 ml ice-cold PBS, centrifuged to yield a TI cell pellet and then lysed in 100 μl of 20 mM Tris containing 5 mM NaCl, 1.8 mM $MgCl_2$ and 1% SDS. The protein level in each sample was evaluated using a BCA Protein Assay kit (Pierce, Rockford, IL) and a DU-650 spectrophotometer (Beckman Coulter). Twenty micrograms of total protein extract was boiled in Laemmli buffer (one part sample Laemmli buffer) for 5 min at 100 °C in a sealed tube before electrophoresis. The samples were subjected to SDS-PAGE (10% Tris–HCl ready gels, Bio-Rad laboratories, Hercules, CA) under reducing conditions. After electrophoresis, proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK); complete transfer was assessed using prestained protein standards (Bio-Rad laboratories, Hercules, CA). The membranes were treated for 12 h with blocking solution (5% non-fat powdered milk in Tris-buffered saline and 0.1% Tween 20) (TBS-T), and then the membranes were incubated for 1 h at room temperature with a primary antibody that reacts with amino acids 421–441 of rat cytochrome P450scc enzyme (Chemicon International, CA). After washing with TBS-T and TBS, membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody, and the resolved P450scc was detected using the enhanced chemiluminescence (ECL) system (Amersham Life Science, Buckinghamshire, UK). Scanning densitometry was used to estimate the pixel density of individual bands using ImageQuant 5.2 Software.

2.9. Statistical analysis

Data were expressed as the mean ± standard error of 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.

3. Results

3.1. Effect of HPTE on progesterone production

To evaluate whether HPTE inhibits progesterone production, TI cells or GC were exposed to HPTE (0,10, 50 or

Table 1
Primer sets used (number in parentheses represents gene accession number)

Gene	Primers	Universal probe
GAPDH (NM_002046)		
Sense	AGC CAC ATC GCT CAG ACA C	#60
Antisense	GCC CAA TAC GAC CAA ATC C	
Cyp11A1 (NM_017286.1)		
Sense	TAT TCC GCT TTG CCT TTG AG	#9
Antisense	CAC GAT CTC CTC CAA CAT CC	
StAR (ENSRNOT00000020606.3)		
Sense	AAG GCT GGA AGA AGG AAA GC	#2
Antisense	CAC CTG GCA CCA CCT TAC TT	
AND (D50436.1)		
Sense	CCT GGC TTT TGG ACT AAC AAA	#26
Antisense	TCC ATA GCC TTG GTC AGA CA	
ADR (ENSRNOT00000004592.3)		
Sense	ATC CTG CTG ACC CCA CCT	#80
Antisense	CCC CAG TGC AAC CTC TGT	

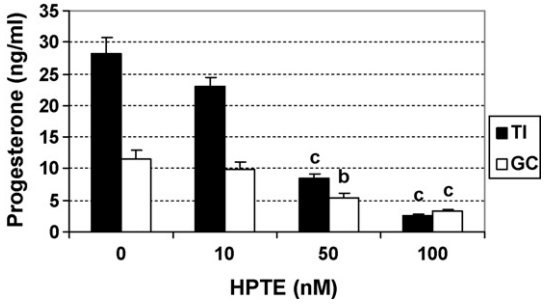

Fig. 1. Effect of HPTE on progesterone formation by TI cells or GC. Cells (1×10^5/ml) were exposed to varying concentrations of HPTE (0, 10–100 nM) at the time of plating for 24 h. HPTE was dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$). (b and c) $P < 0.01$ and $P < 0.001$, respectively, when compared with the appropriate control group.

100 nM) alone for 24 h. Progesterone levels declined progressively from 28.21 ± 2.50 ng/ml/10^5 cells in the control group to 2.51 ± 0.22 ng/ml/10^5 cells (9% of control) in TI cells treated with 100 nM HPTE (Fig. 1). Similarly, GC production of progesterone decreased progressively from 11.42 ± 1.14 ng/ml/10^5 cells in the control group to 3.21 ± 0.32 ng/ml/10^5 cells (28% of control) in cells treated with 100 nM HPTE (Fig. 1).

3.2. Effect of HPTE on P450scc activity of TI cell or GC

To demonstrate the direct effect of HPTE on P450scc activity, TI cells or GC were exposed to HPTE (0, 10, 50, 100 or 500 nM) and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) as described in Section 2 to quantitate the release of ^{3}H-labeled side-chain into the medium. Control cells released 14.48 ± 1.13 ng/16 h/10^5 cells. A significant decline in TI cell P450scc activity was observed following exposure to 50 nM HPTE, which decline further to 24% of control in the cells exposure to 500 nM HPTE (Fig. 2). There was no measurable decline in the P450scc activity of GC following exposure to

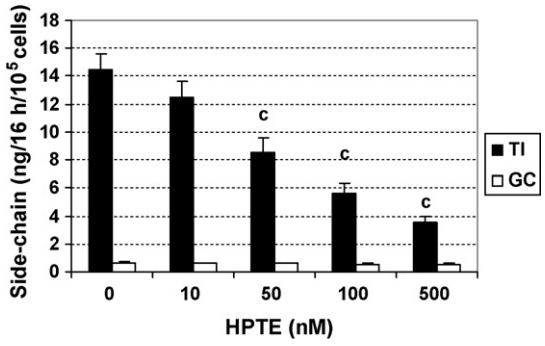

Fig. 2. Effect of HPTE on P450scc activity of TI cells or GC. Cells (2×10^5 for TI or 5×10^5 for GC) were exposed to varying concentrations of HPTE (0, 10–500 nM) for 24 h and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) for the last 16 h. The release of ^{3}H-labeled side-chain (^{3}H-4-hydroxyl-4-methyl-pentanoic acid) into medium was measured. HPTE was dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$). (c) $P < 0.001$ when compared with the TI cell control group.

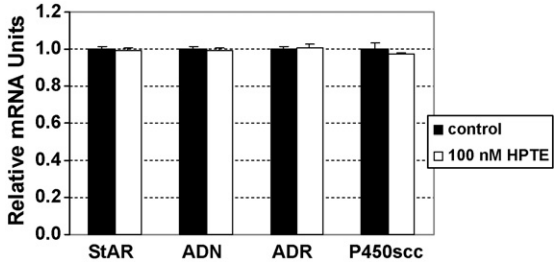

Fig. 3. Effect of HPTE on mRNA levels of P450scc system and StAR. TI cells (3 million) were exposed to HPTE (0 or 100 nM) for 24 h, and the mRNA levels were measured immediately after isolation of TI cells. HPTE was dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from three separate experiments ($n = 3$).

10–500 nM HPTE. Possibly, this was due to the much lower P450scc activity and reduced sensitivity of the assay in GC (Fig. 2). There was about a 21-fold difference between P450scc activities of TI cells (14.48 ± 1.13 ng/16 h/10^5 cells in control) and GC (0.64 ± 0.06 ng/16 h/10^5 cells in control, Fig. 2).

3.3. Effect of HPTE on P450scc gene and protein expression

P450scc must receive electrons from NADPH through the intermediacy of two electron transfer proteins, adrenodoxin reductase (ADR, a flavoprotein) and adrenodoxin (ADN, a iron/sulfur protein) [28] to complete the side-chain cleavage of cholesterol. In addition, a mitochondrial membrane protein, steroidogenic acute regulatory protein (StAR), is involved in delivering cholesterol from the outer to inner mitochondrial membrane where P450scc (CYP11A1) is localized [29]. To evaluate whether HPTE alters mRNA levels of the P450scc system and/or StAR, TI cells were exposed to HPTE (0 or 100 nM) for 24 h, and the mRNA levels for each protein were measured. There was no statistically significant change in the mRNA levels of P450scc, ADN, ADR or StAR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference standard (Fig. 3).

To evaluate whether HPTE alters P450scc protein levels, cultured TI cells were exposed to HPTE (0, 20 or 100 nM) for 24 h, and the P450scc protein levels were measured by immunoblotting methods. P450scc protein levels of TI cells exposed to 20 or 100 nM HPTE were no different than the control level (Fig. 4).

3.4. Effect of estradiol or the xenoestrogens, bisphenol-A or octylphenol, on progesterone formation

Because HPTE binds to the ER and acts as an ERα agonist [20], studies were conducted to determine whether HPTE inhibition of progesterone formation is mediated through the ER pathway. TI cells or GC were exposed to varying concentrations of 17β-estradiol or the weak estrogen agonists, bisphenol-A (constituent in the manufacture of polycarbonate plastics or epoxy resins) or 4-*tert*-octylphenol (a surfactant additive) for 24 h. There was no change in progesterone production by TI cells or GC following exposure to 10–1000 nM 17β-estradiol

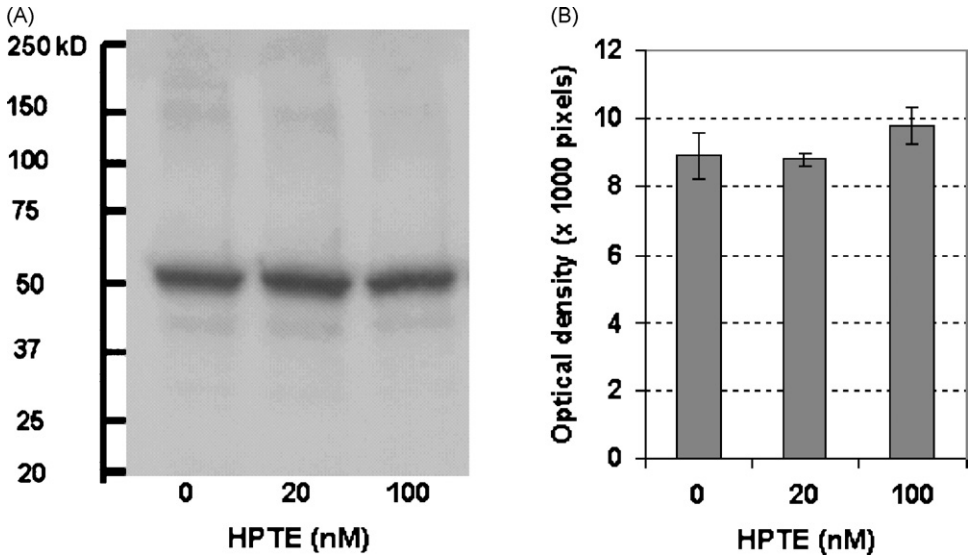

Fig. 4. Western blot detection of P450scc protein in the ovarian TI cells. TI cells were cultured for 24 h in the presence of 0, 20 or 100 nM HPTE. Total protein (20 μg/lane) from each treatment group was resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against rat P450scc. A specific band of ~53-kD in molecular weigh was detected (Panel A). The blot is representative of three separate assays. The amount of P450scc was quantitated by densitometry using ImageQuant 5.2 program (Panel B). Each treatment group represents the mean ± S.E.M. from three separate experiments ($n = 3$).

(24.26 ± 0.88 ng/ml/10^5 cells and 13.64 ± 1.17 ng/ml/10^5 cells in control TI and GC, respectively, Fig. 5, Panel A). Following exposure to 10–1000 nM bisphenol-A, progesterone production in TI cells increased from 26.31 ± 1.62 ng/ml/10^5 cells in the control group to 31.84 ± 1.62 and 30.97 ± 1.56 ng/ml/10^5 cells in cells exposed to 50 or 100 nM bisphenol-A, respectively; however, at the highest concentration of bisphenol-A, TI cell progesterone production was no different than control (Fig. 5, Panel B). GC progesterone level in control cells was 7.52 ± 0.24 ng/ml/10^5 cells, and this was unaffected by exposure to bisphenol-A (Fig. 5, Panel B). Exposure of TI cells to 10–1000 nM octylphenol resulted in statistically significant increases in progesterone levels at the 10 or 50 nM concentrations (from 25.76 ± 1.32 ng/ml/10^5 cells in control cells to 31.92 ± 1.61 and 29.68 ± 1.80 ng/ml/10^5 cells, respectively, Fig. 5, Panel C). Progesterone production of TI cells exposed to 100 or 1000 nM octylphenol was no different than the control level. The progesterone level in control GC was 11.90 ± 0.38 ng/ml/10^5 cells, and progesterone levels were unaffected by exposure to 10–1000 nM octylphenol (Fig. 5, Panel C).

Over all, these studies show that the native estrogen has no effect on progesterone formation by TI or GC and that two xenoestrogenic chemicals, bisphenol-A or octylphenol, actually increased progesterone formation in TI cells at the lower dose range (10–100 nM) used in this study.

3.5. *Effect of antiestrogen alone or with HPTE on TI or GC progesterone production*

The following studies were conducted to determine whether the inhibitive effect of HPTE on TI cell or GC progesterone formation was due to its intrinsic antiestrogenic properties or whether its effect could be blocked by the concomitant inclusion

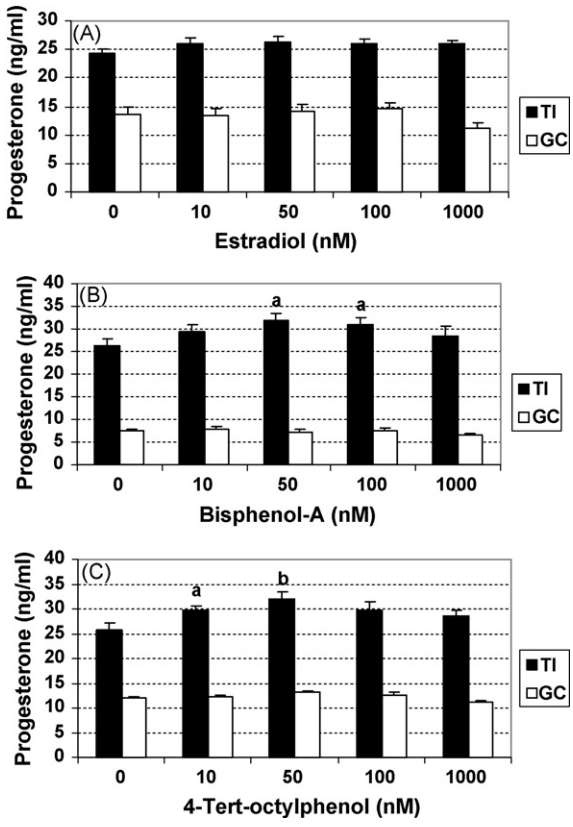

Fig. 5. Effect of estrogen or estrogen agonists on progesterone formation by TI cells or GC. Cells (1×10^5/ml) were exposed to varying concentrations of estradiol, bisphenol-A or 4-*tert*-octylphenol for 24 h. Each chemical was dissolved in ethyl alcohol (100%), and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$). (a and b) $P < 0.050$ and $P < 0.01$, respectively, when compared to the appropriate control group.

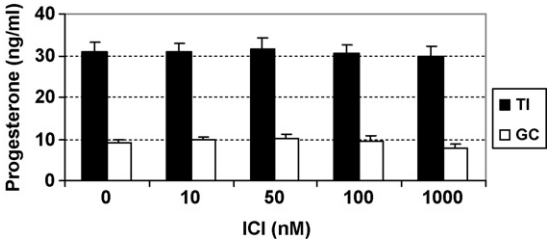

Fig. 6. Effect of the pure estrogen receptor antagonist, ICI 182,780, alone on progesterone production by TI cells or GC. Cells (1×10^5/ml) were exposed to varying concentrations of ICI for 24 h. ICI was dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$).

of the "pure" estrogen antagonist ICI 182,780 (ICI) which binds to both ERα and ERβ [30].

TI cells or GC were exposed to varying concentrations of ICI (0, 10–1000 nM) alone for 24 h. Progesterone levels in control TI cells and GC were 30.76 ± 2.52 ng/ml/10^5 cells and 9.08 ± 0.86 ng/ml/10^5 cells, respectively (Fig. 6). Exposure to ICI alone had no effect on progesterone production in either TI cells or GC.

In a follow-up study, TI cells or GC were concomitantly exposed to varying concentrations of HPTE (0–100 nM) and/or ICI (5 μM) for 24 h. The progesterone level in control TI cells was 24.13 ± 1.37 ng/ml/10^5 cells (Fig. 7). Exposure to HPTE inhibited progesterone formation, which declined progressively to 2.24 ± 0.20 ng/ml/10^5 cells (9% of control) following exposure to 100 nM HPTE. In GC, progesterone production declined from 10.89 ± 1.47 ng/ml/10^5 cells in control cells to 1.39 ± 0.38 ng/ml/10^5 cells (13% of control) following exposure to 100 nM HPTE. Thus, the concomitant exposure to ICI did not alter the inhibitive effects of HPTE on TI cell or GC progesterone formation (Fig. 7).

3.6. Effect of antiandrogenic chemicals on progesterone formation by TI or GC

To evaluate whether the inhibition of progesterone production by HPTE in ovarian cells was due to its antiandrogenic properties, TI cells or GC were exposed to the antiandrogens flutamide or the vinclozolin metabolite, M-2. Vinclozolin is a fungicide currently used on various fruits and vegetables. Flutamide is an oral antiandrogen drug primarily used to treat prostate cancer. However, recent studies suggested that flutamide may also have antiprogestogenic activity [31].

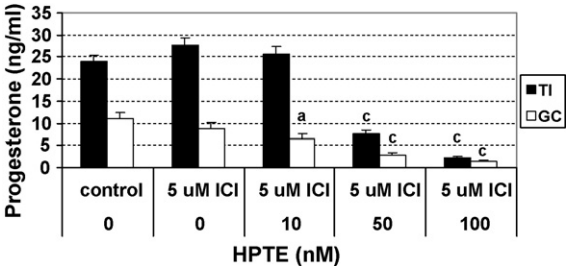

Fig. 7. Effect of concomitant exposure to ICI and HPTE on progesterone formation by TI cells or GC. Cells (1×10^5/ml) were exposed to ICI (0 or 5 μM), and varying concentrations of HPTE (0, 10–100 nM) for 24 h. HPTE and ICI were dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$). (a and c) $P < 0.050$, and $P < 0.001$, respectively, when compared to the appropriate control group.

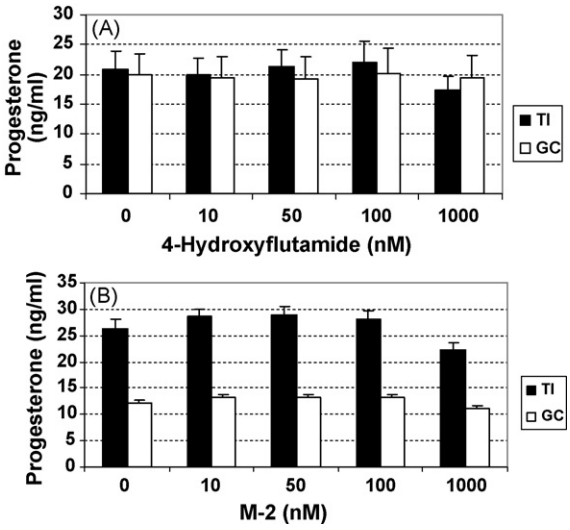

Fig. 8. Effect of androgen antagonists on progesterone formation by TI cells or GC. Cells (1×10^5/ml) were exposed to varying concentrations of 4-hydroxyflutamide alone or the vinclozolin metabolite, M-2, alone for 24 h. These compounds were dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments ($n = 3$).

Progesterone levels in control TI and GC were 20.79 ± 3.11 and 19.81 ± 3.53 ng/ml/10^5 cells, respectively (Fig. 8, Panel A). Exposure to flutamide alone (10–1000 nM, Fig. 8, Panel A) had no effect on progesterone formation. Similarly, exposure to M-2 alone (10–1000 nM) had no effect on progesterone production by TI cells or GC (Fig. 8, Panel B). Collectively, these results suggest that the inhibition of progesterone formation in TI cells or GC by HPTE is not due to its antiandrogenic properties.

4. Discussion

Data obtained in this study indicate that the active metabolite of MC, HPTE, directly inhibits progesterone formation by cultured TI and GC from PMSG-primed immature rats and that this decline in progesterone appears to be correlated to a decline in the catalytic activity of P450scc, at least in TI cells. This effect of HPTE on P450scc activity, which converts cholesterol to pregnenolone in the pathway of progesterone and estrogen biosynthesis, could be observed at 50 nM in TI cells. The inhibition of P450scc activity by HPTE suggests that this is a direct effect on the enzyme, which is also confirmed by unchanged P450scc protein levels and mRNA levels of the three components of the P450scc system (P450scc, ADR and ADN) in TI cells following exposure to 100 nM HPTE. Because the concomitant exposure to the "pure" antiestrogen,

ICI, with HPTE did not alter the pattern of response to HPTE, and none of various test compounds acting as an estrogen (17β-estradiol, bisphenol-A or octylphenol), antiestrogen (ICI) or antiandrogen (flutamide or the vinclozolin metabolite, M-2) and added alone to TI cells or to GC, mimicked the action of HPTE on progesterone production, these results suggest that the effects of HPTE on TI cell or GC progesterone formation are not due to its estrogenic, antiestrogenic or antiandrogenic properties.

The current observations suggest that HPTE directly inhibits P450scc catalytic activity, which, in turn, is responsible for a decline in progesterone formation by TI cells. Although the present studies did not detect a decline in P450scc activity in GC in response to HPTE, it is possible that this was due to the lower activity of the enzyme and reduced sensitivity of the assay in GC. Alternatively, HPTE may inhibit progesterone formation in GC at a locus other than the P450scc site.

The inhibitive effect of HPTE on P450scc activity in TI cells is in agreement with previous observations that the dose-dependent decline in testosterone formation in rat Leydig cells correlated with a similar pattern of decline in the activity of this enzyme [21–23]. Although how HPTE specifically inhibits this step is not known, a possible mode of HPTE action is to occupy the cholesterol-binding region of P450scc. The conversion of cholesterol to pregnenolone involves three distinct steps: 20α-hydroxylation, 22-hydroxylation and the scission of the C20–22 carbon bond, and these steps occur within the single substrate-binding pocket of P450scc [28]. To our knowledge, there has been no study showing that HPTE covalently binds to P450scc, but HPTE was reported to bind covalently to liver microsomal proteins in the presence of NADPH [32]. Moreover, the parent compound MC was shown to inhibit P450scc activity of bovine adrenocortical mitochondria by binding near to the cholesterol-binding region of this enzyme [33]. Another possible mode of action of HPTE is to alter the electron transport system. P450scc must receive electrons from NADPH through the intermediacy of two electron transfer proteins, adrenodoxin reductase (ADR, a flavoprotein) and adrenodoxin (ADN, a iron/sulfur protein) [28]. It has been reported that the covalent binding of MC to liver microsomal proteins involves the generation of several reactive oxygen species [34], which may damage enzymes involved in steroidogenesis. It was reported that inclusion of antioxidants had a protective effect against MC binding to microsomal liver proteins [32]. However, Murono et al. reported that the concomitant exposure to increasing concentrations of two different antioxidants (trolox or ascorbate) did not reverse the inhibitive effects of HPTE on androgen production by rat Leydig cells [21,23].

Although our current studies found no effect of 100 nM HPTE on P450scc mRNA levels in TI cells, a recent study in cultured rat GC reported that FSH-stimulated P450scc mRNA levels were reduced following exposure to 10 μM HPTE for 2 days [35]. In this study, lower concentrations of HPTE (0.1 or 1 μM) were without effect [35]. In addition, the mRNA levels of 3β-hydroxysteroid dehydrogenase and P450 aromatase also were reduced in rat GC following treatment with 10 μM HPTE [35]. These opposing results could be explained by differences in the concentrations of HPTE used, differences in exposure time or differences in culture conditions used. It should be noted that under the present culture conditions, there was a tendency for both TI cells and GC to detach from the culture wells with HPTE concentrations of over 1 μM (data not shown). A study by Akingbemi et al. reported that the inhibition of testosterone production by rat Leydig cells following exposure to HPTE was due to a decline in P450scc activity, which was the result of the decline of P450scc mRNA levels [24]. This suggests that enzyme activity declined due to reduced protein synthesis. However, in the current study mRNA levels of P450scc in TI cells were not affected by HPTE, although progesterone levels and P450scc activity declined. It was also reported that HPTE inhibited androgen formation by cultured Leydig cells as early as 1 h following to exposure HPTE [23]. In ovarian cells, we observed the inhibition of progesterone production by HPTE within 3 h (unpublished observation). These reasonably rapid effects of HPTE on steroid production suggest that the inhibition of new protein synthesis may not explain how P450scc activity is suppressed in steroidogenic cells by HPTE because the half life of P450scc is estimated to be greater than 4 h [36]. Although the decline in progesterone formation in rat TI cells appears to be due mainly to a direct effect of HPTE on P450scc activity, it is possible also that HPTE could act partly by increasing P450scc mRNA degradation and/or accelerating progesterone metabolism.

Although there is general agreement that HPTE inhibits the P450scc step, the mechanism of action is not established. Because HPTE acts as an ERα agonist but is antagonistic to ERβ and AR, the receptor-linked actions of HPTE are plausible [20]. To evaluate if the inhibition of progesterone production by HPTE is through a receptor-mediated pathway, various estrogenic, antiestrogenic and antiandrogenic compounds were exposed to TI cells. These compounds are known to strongly bind steroid receptors but their effects on progesterone synthesis are not established. Some of them may also bind multiple steroid receptor types. For example the antiandrogenic drug flutamide was reported to block progesterone-induced uterine CaBP-9k mRNA and protein expression in mice [31]. In the current study none of the estrogenic agents including 17β-estradiol itself, or the xenoestrogenic agents, bisphenol A or octylphenol, inhibited progesterone formation by TI cells or GC. In fact, bisphenol A or octylphenol modestly increased progesterone formation in TI cells, and when the "pure" estrogen antagonist ICI was added concomitantly with HPTE, it did not alter the inhibition of HPTE on progesterone formation. Similarly, the antiandrogenic agents, flutamide or M-2, had no effect on progesterone production by TI cells or GC. Collectively, these studies suggest that a receptor-mediated action of HPTE is unlikely. In agreement with our current results, a recent study also showed that exposure to estrogenic, antiestrogenic or antiandrogenic compounds did not alter androgen production by cultured rat Leydig cells [21], and the concomitant inclusion of ICI with HPTE did not block the inhibitive effects of HPTE. In contrast, another study suggested that the inhibition of rat Leydig cell androgen production by HPTE was mediated through the ER pathway based on the observation that the concomitant inclu-

sion of ICI blocked the effects of HPTE [24]. We are unable to explain these differences in the results with ICI at the present time.

In conclusion, this study demonstrates that HPTE directly inhibits progesterone formation in both cultured rat TI and GC and that this is correlated with a decline in P450scc catalytic activity at least in TI cells. This action of HPTE does not appear to be mediated through the ER or AR signaling pathways, the currently recognized modes of action of MC and HPTE. In addition, the inhibition of progesterone formation in TI cells or GC by HPTE does not appear to be due to its estrogenic, antiestrogenic or antiandrogenic properties.

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