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The methoxychlor metabolite, HPTE, inhibits rat luteal cell progesterone production☆

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ABSTRACT

The methoxychlor metabolite, HPTE, was shown to inhibit P450-cholesterol side-chain cleavage (P450scc) activity resulting in decreased progesterone production by cultured ovarian follicular cells in previous studies. It is not known whether HPTE has any effect on progesterone formation by the corpus luteum.

Results: Exposure to 100 nM HPTE reduced progesterone production by luteal cells with progressive declines to <22% of control at 500 nM HPTE. Similarly, HPTE progressively inhibited progesterone formation and P450scc catalytic activity of hCG- or 8 Br-cAMP-stimulated luteal cells. However, HPTE did not alter mRNA and protein levels of P450scc. Compounds acting as estrogen (17β-estradiol, bisphenol-A or octylphenol), antiestrogen (ICI) or antiandrogen (monobutyl phthalate, flutamide or M-2) added alone to luteal cells did not mimic the action of HPTE on progesterone and P450scc activity. These results suggest that HPTE directly inhibits P450scc catalytic activity resulting in reduced progesterone formation, and this action was not mediated through estrogen or androgen receptors.

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1. Introduction

Environmental or occupational exposure to endocrine-disrupting chemicals (EDCs) has been reported to alter the normal functioning of the endocrine system in humans and wildlife [1,2]. One example of an environmental toxicant is the recently banned pesticide methoxychlor (MC) [3], which is released into the environment as a result of its applications to agricultural crops, livestock, animal feed, stored grains, home gardens, and pets [4]. *In vivo*, MC alters reproductive and ovarian function in exposed laboratory rodents. Some of the reported adverse effects include embryonic toxicity [5], precocious puberty [6], decreased fertility [7], impaired testicular steroidogenesis [8–11], ovarian atrophy [12–14] and a dose-dependent decline in the number of follicles and ovarian steroid production [15–18]. It is generally thought 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) [19]. In the female rodent, MC or HPTE causes an uterotropic response and proliferation of ovarian surface epithelium through estrogen receptor (ER)-linked mechanisms [20–22]. Both MC and HPTE have weak estrogenic and antiandrogenic activities [23–25]. However, HPTE binds with higher affinity to the ER [19], and it is a more potent androgen receptor (AR) blocker [25]. In human hepatoma cells (HepG2), HPTE acted as an ERα agonist, but it was antagonistic toward ERβ and AR [26]. Although it generally is thought that chemicals such as HPTE exert their effects through receptor-mediated pathways, recent studies have suggested that some actions of HPTE on steroidogenesis may not be mediated through the ER or AR [9,18]. For example, studies by our group demonstrated that the concomitant addition of the "pure" antiestrogen, ICI 182,780, did not block inhibition of rat Leydig cell androgen formation nor progesterone production of ovarian theca cells by HPTE [9,18]. These studies also demonstrated that the exposure to the antiandrogenic chemicals, hydroxyflutamide or vinclozolin, did not affect androgen production of Leydig cells or progesterone production of theca cells

Previous *in vivo* studies in female and male rodents demonstrated decreased serum progesterone and androgen levels, respectively, following exposure to MC [7,8,17]. The effects of HPTE on ovarian and testis steroidogenesis were localized to the mitochondrial P450 cholesterol side-chain cleavage (P450scc) step which converts cholesterol to pregnenolone in the pathway of androgen and estrogen production [8–11,18]. Our previous studies

☆ These studies were conducted in partial fulfillment for a Ph.D. at W.V.U. by Y.A.

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demonstrated that HPTE inhibited the catalytic activity of P450scc resulting in decreased progesterone formation in follicular cells (theca-interstitial and granulosa) [18]. It has not been established whether HPTE has similar effects on steroid production in the corpus luteum. After ovulation follicular cells differentiate into non-dividing progesterone-producing luteal cells [27]. The current studies examined the effect of HPTE on progesterone production by cultured luteal cells from immature rats treated with an ovulatory dose of pregnant mare serum gonadotropin (PMSG).

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, 3–4 pups were placed in a polycarbonate shoebox cage containing a mixture of Alphadri (Shepard 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), 22(R)hydroxycholesterol, 5-pregnen-3β-ol-20-one (pregnenolone), 8-bromoadenosine 3′-5′-cyclic monophosphate (8 Br-cAMP) 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), 4-*tert*-octylphenol (octylphenol) and monobutyl phthalate (MBP) were from Aldrich Chem. Co., Milwaukee, NY. 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2, 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 ovulatory dose of PMSG (20 IU/0.1 ml, s.c.) dissolved in PBS. Animals were treated between 0800 and 1000 h each day. Animals ovulate about 72 h following PMSG injection, and this day was marked as post ovulation day 0. Following ovulation the ruptured follicles are converted into corpora lutea, which have a functional life span of 10–12 days [28].

2.4. Isolation and incubation of luteal cells

Luteal cells were isolated by slight modifications of previously described methods for theca-interstitial cells [18]. Briefly, the ovaries were harvested 5 days following the anticipated day of ovulation. They were stripped of bursa and fat tissue and then punctured several times with a 26-gauge needle until all antral follicles had been pierced to release granulosa cells, while corpora lutea remained intact. The ovarian tissue was washed twice with medium and incubated 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, and the remaining tissue was further digested with collagenase for an additional 50 min to disperse the luteal tissue. Ice-cold Medium 199 was added to stop further dissociation, and the undigested tissue was allowed to settle to the bottom of the 50 ml conical tubes and discarded. The supernatant containing dispersed luteal cells was aspirated and centrifuged (209 × *g*) for 10 min to yield a luteal cell pellet. The final pellet was then resuspended in DMEM/F-12 medium.

Luteal cells (10^5/well) were plated into 24-well 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 as described previously [29]. Cells were exposed to concentrations of 0, 10, 50, 100 or 500 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, incubation period, luteal cell age and luteal cell numbers per well were chosen based on preliminary studies conducted to determine the sensitivity of cultured luteal cells to the chemical. 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 cell to culture wells.

2.5. Measurement of progesterone

Progesterone was quantitated directly from the collected medium by radioimmunoassay (RIA) 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 luteal 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 [30] with slight modifications. In brief, following isolation of luteal cells (1×10^5/0.5 ml medium), cells were plated in 24-well culture plates. Varying concentrations of HPTE (0, 20, 100 or 500 nM) were added at the time of plating and cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 h. Following washing the cells twice with fresh medium, the cells were retreated with corresponding HPTE concentrations, and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) was added to each well. The cells were incubated for an additional 4 h at 37 °C. Reactions were stopped by adding 50 μl of 1 N 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 of PBS. The PBS washes were transferred into 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. 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 Luteal cells (∼3 million) cultured in the absence or presence of 500 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) [31]. Briefly, the method involves obtaining the C_T values for the genes of P40scc (*Cyp11a1*), 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD Type I, *Hsd3b1*), adrenodoxin (*Adn*) and adrenodoxin reductase (*Adr*), normalizing to a reference gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and deriving the fold increase compared to the control group (Table 1).

2.8. Western blot analysis

Luteal 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 [18]. Cells were exposed to concentrations of 0 or 500 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 then were scraped off the dishes using a cell lifter, and the cells were resuspended in 20 ml ice-cold PBS, centrifuged to yield a luteal 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). 20 μg of total protein extract was boiled in Laemmli buffer (1 part sample and 1 part 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

Table 1
Primer sets for RT-QPCR (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	Sense	TAT TCC GCT TTG CCT TTG AG	#9
(NM_017286.1)	Antisense	CAC GAT CTC CTC CAA CAT CC	
Adn (D50436.1)	Sense	CCT GGC TTT TGG ACT AAC AAA	#26
	Antisense	TCC ATA GCC TTG GTC AGA CA	
Adr	Sense	ATC CTG CTG ACC CCA CCT	#80
(ENSRNOT00000004592.3)	Antisense	CCC CAG TGC AAC CTC TGT	
Hsd3b1 (NM_017265.4)	Sense	TCA GAC CAG AAA CCA GAG AAG AA	#12
	Antisense	AGG CAC TGG GTG TCA AGA AT	

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 One-way 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. Basal, hCG or 8 Br-cAMP-stimulated luteal cell progesterone production is progressively inhibited by HPTE

Studies were initiated to determine whether HPTE affects basal, hCG (10 mIU/ml) or 8 Br-cAMP (0.1 mM) stimulated progesterone formation by luteal cells. The basal progesterone level following 24 h of culture was 13.71 ± 1.93 ng progesterone/10^5 cells (Fig. 1). A statistically significant decline in progesterone was observed with 100 nM HPTE, which declined further to 3.04 ± 1.09 ng/ml/10^5 cells (22% of control) following exposure to 500 nM HPTE for 24 h. Similarly, hCG-stimulated levels of progesterone declined from 27.24 ± 3.22 ng/ml/10^5 cells in control cells to 11.13 ± 3.78 ng/ml/10^5 cells (41% of control) in cells exposed to 500 nM HPTE (Fig. 1). The cAMP-stimulated cells also showed dose dependent inhibition of progesterone production by HPTE as a 74% reduction from 28.53 ± 1.41 ng/ml/10^5 cells in control cells was recorded in the cells exposed to 500 nM HPTE (Fig. 1).

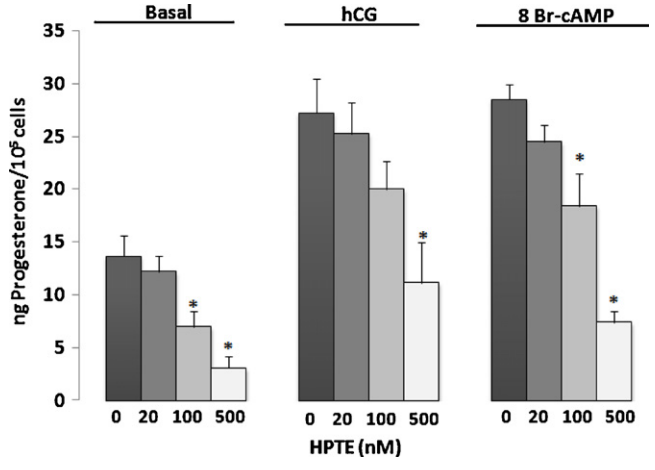

Fig. 1. Effect of HPTE on basal, hCG-stimulated or 8 Br-cAMP-stimulated luteal cell progesterone. Luteal cells (1×10^5/ml) were exposed to varying concentrations of HPTE alone (0, 20, 100 or 500 nM) or HPTE (0, 20, 100 or 500 nM) and 10 m IU/ml hCG or HPTE (0, 20, 100 or 500 nM) or 0.1 mM 8 Br-cAMP at the time of plating for 24 h. Each treatment group represents the mean ± S.E.M. of four samples from at least five separate experiments. *P < 0.05 when compared to the appropriate control group.

3.2. ER or AR binding compounds do not affect progesterone formation by luteal cells

Because, HPTE has been shown to act as an ERα agonist, ERβ antagonist and AR blocker, studies were conducted to determine whether HPTE action is mediated through the ER or AR pathway to inhibit progesterone [26].Luteal cells were treated for 24 h with vehicle or either one of 500 nM of the steroid receptor binding chemicals. The chemicals tested were HPTE, 17β-estradiol (E2), xenoestrogen bisphenol-A (Bis A) and 4-tert-octylphenol (OP), antiandrogen monobutyl phthalate (MBP), M2 or hydroxy flutamide (OH-Flu), and pure antiestrogen ICI (blocks both ER α and β). Of each of the chemicals tested only HPTE caused a reduction (about 50%) in luteal progesterone production (Fig. 2).

3.3. Inhibition of HPTE on luteal cell progesterone formation is not altered by "pure" antiestrogen ICI 182.780

The aim of the studies was to determine whether the inhibition of HPTE on luteal cell progesterone production was due to its intrinsic antiestrogenic properties or whether its effect could be blocked by concomitant inclusion of the "pure" antiestrogen ICI 182,780 which binds to both ERα and ERβ [32]. Luteal cells

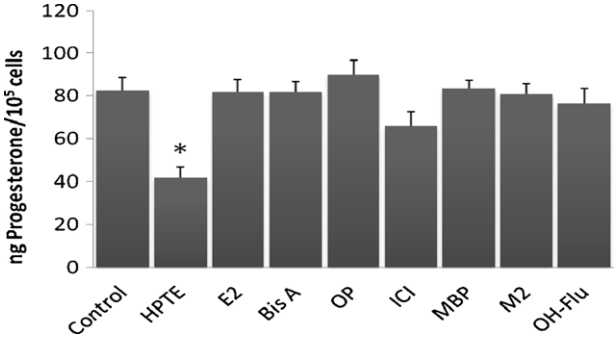

Fig. 2. Effect of various estrogenic (E2, Bis A and OP), antiestrogenic (ICI) or antiandrogenic (MBP, M2 and OH-Flu) chemicals on luteal cell progesterone. Luteal cells (1×10^5/ml) were exposed to 500 nM of one of the chemicals at the time of plating for 24 h. Each treatment group represents the mean ± S.E.M. of four samples from at least four separate experiments. *P < 0.05 when compared to the appropriate control group.

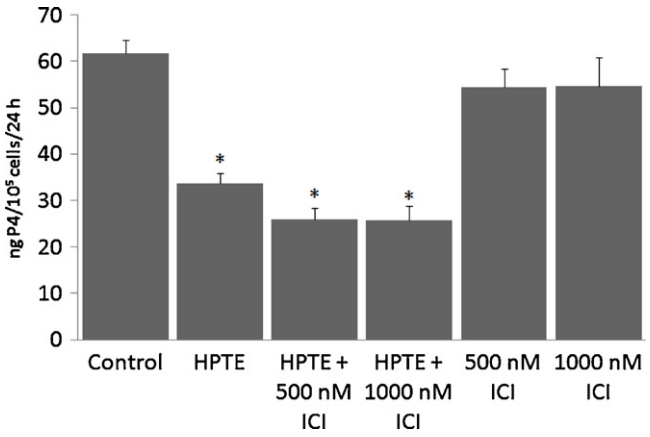

Fig. 3. Effect of ICI on HPTE inhibited luteal cell progesterone formation. Cells (1×10^5/ml) were exposed to varying concentrations of ICI (0, 0.5 or 1 μM), and/or HPTE (0.5 μM) for 24 h. HPTE and ICI were dissolved in DMSO, and all treatment groups contained 0.1% of the vehicle. Each treatment group represents the mean ± S.E.M. of four samples from at least four separate experiments ($n = 4$). *$P < 0.05$ when compared to the control group.

were exposed to HPTE (0 or 500 nM) alone or varying concentrations of ICI (0.5 or 1 μM) alone, or concomitantly exposed to HPTE (500 nM) and ICI (0.5 or 1.0 μM) for 24 h. Progesterone levels declined from 61.72 ± 2.94 ng/ml/10^5 cells in control group to 33.67 ± 2.15 ng/ml/10^5 cells (55% of control) in luteal cells treated with 500 nM HPTE. Similarly, HPTE and 0.5 or 1.0 μM ICI co-treated cells produced 26.01 ± 2.3 ng/ml/10^5 cells (42.2% of control) or 25.73 ± 3.07 ng/ml/10^5 cells (42.2% of control), respectively (Fig. 3). Exposure to 500 or 1000 nM ICI alone had no effect luteal cell progesterone production. Thus, the concomitant exposure to ICI did not alter the inhibition of HPTE on luteal cell progesterone formation (Fig. 3).

3.4. HPTE inhibits P450scc step but not 3β-HSD step of luteal steroidogenesis

To evaluate whether exposure to HPTE inhibits steroidogenic enzymes involved in the conversion of cholesterol to progesterone, luteal cells were exposed to HPTE (0, 20, 100 or 500 nM) alone for 24 h. Following treatment, fresh medium was added, and cells were co-treated with appropriate concentrations of HPTE (0, 20, 100 or 500 nM) and 22(R)OH-cholesterol (10 μM) for 4 h or pregnenolone (10 μM) for 1 h (Fig. 4). In response to addition of 22(R)OH-cholesterol which provides an indirect measure of P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) activities, progesterone formation decreased to 53% of control following exposure to 500 nM HPTE (Fig. 4, panel A). In contrast, the conversion of pregnenolone to progesterone was unaffected by exposure to HPTE, suggesting HPTE specifically inhibits P450scc step of ovarian steroidogenesis (Fig. 4, panel B). Our previous studies demonstrated similar effects in follicular cells [18] and in testicular Leydig cells [8].

3.5. HPTE inhibits the catalytic activity of P450scc

To demonstrate the direct effect of HPTE on P450scc activity, luteal cells were exposed to HPTE (0, 20, 100 or 500 nM) and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) as described in the methods section to quantitate the release of ^{3}H-labeled side-chain into the medium. Control cells released 13.02 ± 1.18 ng side-chain/4 h/10^5 cells. A significant decline in luteal cell P450scc activity was observed following exposure to 500 nM HPTE (45% of control). Studies also were conducted to determine whether HPTE

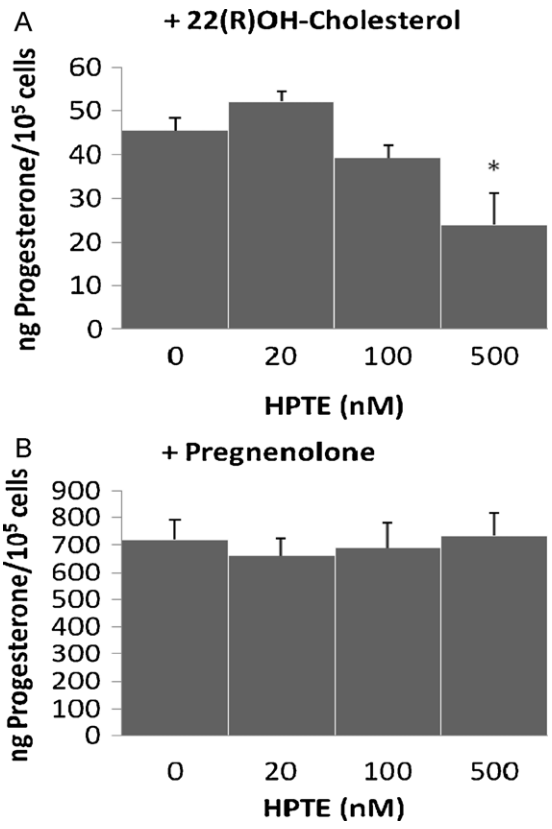

Fig. 4. Effects of HPTE on the conversion of steroid precursors to progesterone. Luteal cells (1×10^5/ml) pre-treated with varying concentrations of HPTE (0, 20, 100 or 500) for 24 h, were then treated with appropriate concentrations of HPTE + 10 μM 22(R)OH-cholesterol for 4 h or HPTE + 10 μM pregnenolone for 1 h. The reagents 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 ($n = 3$). *$P < 0.05$ when compared to the control group.

inhibits P450scc activity of 8 Br-cAMP-stimulated cells. In response to exposure to HPTE (0, 20, 100 or 500 nM) and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) and 8 Br-cAMP (0.2 mM) for 4 h, a significant decline in side-chain was observed at 500 nM HPTE (45% of control, Fig. 5).

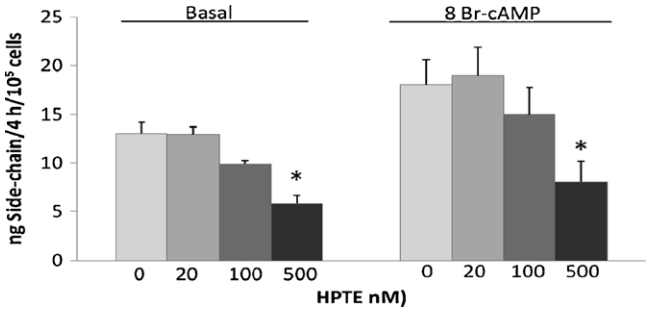

Fig. 5. Effect of HPTE on P450scc activity of rat luteal cells. Luteal cells (1×10^5/0.5 ml) were exposed to varying concentrations of HPTE (0, 10–500 nM) for 24 h then re-treated with appropriate concentrations of HPTE and 25-[26,27-^{3}H]-hydroxycholesterol (0.5 uCi, 5 uM) in the absence or presence of 8 Br-cAMP (0.2 mM) for 4 h. The release of ^{3}H-labeled side-chain (^{3}H-4-hydroxyl-4-methyl-pentanoic acid) into medium was measured. All treatment groups contained 0.1% of the vehicle (DMSO). Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments. *$P < 0.05$ when compared to the control group.

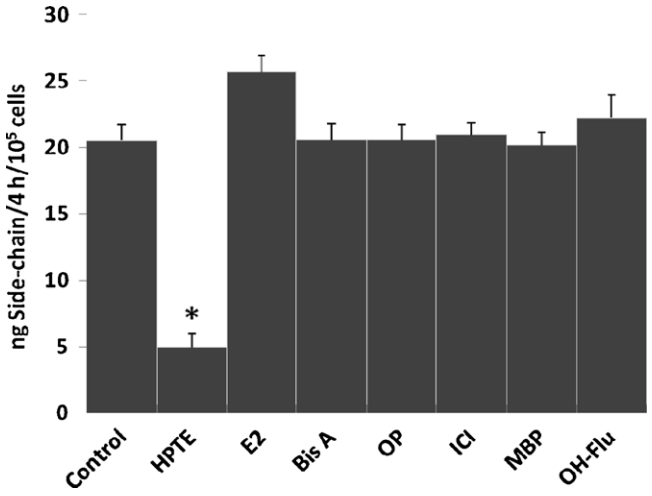

Fig. 6. Effect of various estrogenic (E2, Bis A and OP), antiestrogenic (ICI) or antiandrogenic (MBP, M2 and OH-Flu) chemicals on luteal cell P450scc activity. Luteal cells ($1x10^5$/ml) were concomitantly treated with 25-[26,27-^{3}H]-hydroxycholesterol and one of the chemicals for 4 h following an initial 24 h exposure. Each treatment group represents the mean ± S.E.M. of four samples from at least four separate experiments. $^*P < 0.05$ when compared to the appropriate control group.

3.6. P450scc inhibition by HPTE is unique among steroid receptor binding compounds

To confirm that inhibition of HPTE on P450scc activity is not mediated through ER or AR binding, luteal cells were co-treated 4 h with 25-[26,27-^{3}H]-hydroxycholesterol (0.5 μCi, 5 μM) and 500 nM of one of the steroid receptor binding chemicals, HPTE, E2, Bis A, OP, ICI, MBP, M2, or OH-Flu. Control cells produced 20.48 ± 1.22 ng side-chain/4 h/10^5 cells. A statistically significant decline in side-chain was observed with 500 nM HPTE (24% of control). None of the other chemicals tested had any effect on P450scc activity (Fig. 6).

3.7. HPTE does not alter the gene or protein expression of P450scc

Progesterone production from cholesterol is achieved in two steps, P450scc and 3β-HSD. 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) [33] to complete the side-chain cleavage of cholesterol. To evaluate whether HPTE alters mRNA levels of the P450scc system and/or 3β-HSD, luteal cells were exposed to HPTE (0 or 500 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 (*Cyp11a1*), *Adn*, *Adr* or 3β-HSD (*Hsd3b1*) (Fig. 7, Panel A). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the reference standard.

To evaluate whether HPTE alters P450scc protein levels, cultured luteal cells were exposed to HPTE (0, 500 nM) for 24 h, and the P450scc protein levels were measured by immunoblotting methods. P450scc protein levels of luteal cells exposed to HPTE were no different than the control level (Fig. 7, Panel B).

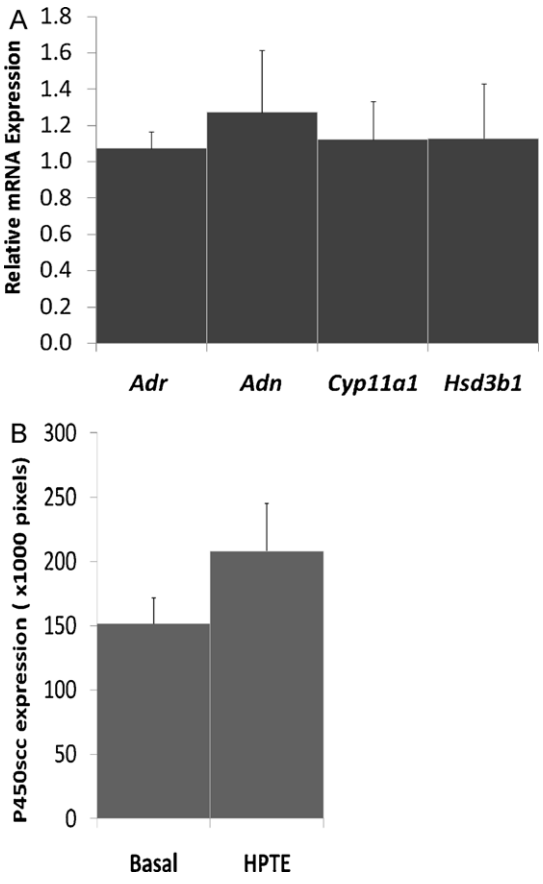

Fig. 7. Effect of HPTE on gene (*P450scc, Adr, Adn and 3β-Hsd*) and protein (P450scc) expression. Each treatment group represents the mean ± S.E.M. of four samples from at least three separate experiments.

4. Discussion

The current studies demonstrate that the active metabolite of MC, HPTE, progressively inhibits progesterone formation by cultured luteal cells from PMSG-treated immature rats. This decline in progesterone formation following exposure to HPTE was associated with a corresponding progressive decline in P450scc activity, a rate-limiting step in the biosynthesis of progesterone, androgens and estrogens. Inhibition of progesterone formation could be observed at 100 nM HPTE. Similarly, HPTE inhibited progesterone formation of hCG- or 8 Br-cAMP-stimulated luteal cells suggesting that HPTE does not inhibit progesterone formation by disrupting the gonadotropin signaling pathway upstream of cAMP step. This also suggest that one or more steps within the cAMP-dependent pathway, following the generation of cAMP (e.g., cAMP phosphodiesterase, PKA, CREB, etc.) may be impeded by HPTE (Fig. 8). The conversion of 22-hydroxycholesterol but not pregnenolone to progesterone is inhibited by HPTE suggesting that the effect is localized to P450scc step. Furthermore, 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 three components of the P450scc system (P450scc, ADR and ADN) in luteal cells following exposure to 500 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 (E2, Bis A or OP), antiestrogen (ICI) or antiandrogen (MBP, OH-Flu or M2) added alone to luteal cells, mimicked the action of HPTE on progesterone production and side-chain cleavage activity, one can conclude that the effects of HPTE on luteal cell progesterone formation are not due to its estrogenic, antiestrogenic or antiandrogenic properties.

The pattern of inhibition by HPTE on progesterone formation by the luteal cells is similar to the pattern observed previously in follicular cells [18], suggesting that the luteinization of follicular cells does not alter effects of HPTE. The corpus luteum expresses high levels of key proteins involved in the uptake and transport of cholesterol which facilitates progesterone production [27]. In fact, the current study demonstrated that the capacity of luteal

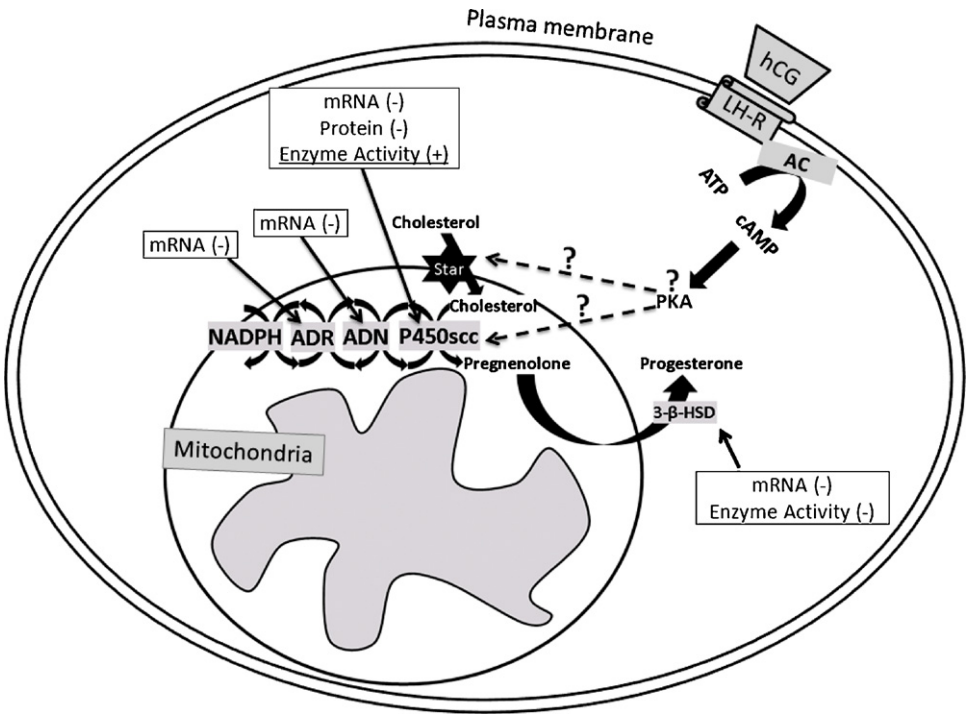

Fig. 8. Effects of HPTE on luteal cell gonadotropin signaling pathway. The effects of HPTE on gene expression, protein level and enzyme activity of the steroidogenic factors are shown in the text boxes based on the current study. The dotted lines demonstrate multiple steps that are downstream of PKA in cAMP dependent gonadotropin signaling. (?), represent unknown effects of HPTE on protein kinase A (PKA) and/or following steps that results in the inhibition P450scc activity that requires electron transfer from NADPH and cholesterol transportation by steroidogenic acute regulatory protein (star) [33].

cells to produce progesterone (up to 80 ng/ml/105 cells) is about 4-fold higher than the capacity of theca interstitial cells and about 10-fold higher than that of granulosa cells under similar culture conditions [18]. Although luteal cells have this enhanced capacity to produce progesterone, exposure to 500 nM HPTE lowered progesterone output to 22% of control in luteal cells (isolated on day 5 post ovulation). However, the inhibition of HPTE was greater in follicular cells compare to luteal cells as a lower concentration of HPTE (100 nM) inhibited progesterone formation to 9% of control in theca interstitial cells of antral follicules, suggesting that more HPTE is required to inhibit elevated progesterone producing capacity of luteinized cells. Our preliminary studies have shown that the sensitivity of luteal cells to HPTE is higher during the early luteal phase (post ovulation days 2 and 5). However, in luteal cells isolated during the later luteal period (post ovulation day 8), progesterone formation was less sensitive to higher concentrations of HPTE (data not shown).

The current study on rat luteal cells confirms previous reports that the dose-dependent decline in progesterone and testosterone formation in rat follicular and Leydig cells, respectively, correlated with a similar pattern of decline in the activity of P450scc [8–10,18]. Thus, HPTE appears to reduce steroid formation in different steroid-producing cells at a common steroidogenic step. Previous studies in rat Leydig cells [11] and in rat ovarian granulosa cells [34] suggested that the decline in P450scc activity was due to the inhibition of new protein synthesis for the enzyme; however, in rat ovarian theca interstitial cells and luteal cells, HPTE had no effect on P450scc mRNA and protein levels. We propose that similar to theca interstitial cells, MC/HPTE inhibits progesterone formation in rat luteal cells by directly inhibiting P450scc catalytic activity.

Although how HPTE specifically inhibits P450scc 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 [33]. In preliminary experiments of the current studies, we observed a decrease in sensitivity to HPTE of luteal cells as pregnancy advanced, suggesting that competitive inhibition by HPTE to the cholesterol-binding site of P450scc might be a mechanism to inhibit the enzyme as suggested previously in bovine adrenocortical cells [35]. However, the addition of exogenous 22-hydroxycholesterol to luteal cells in the current study as well as our previous studies with testicular cells [10] did not block the inhibition of progesterone formation (testosterone in testicular cells) elicited by HPTE. 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 [36]. 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 [35]. 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) [33]. It has been reported that the covalent binding of MC to liver microsomal proteins involves the generation of several reactive oxygen species [37], 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 [36]. However, Murono et al. reported that the concomitant exposure to increasing concentrations of two different antioxidants (trolox or ascorbate) did not reverse the inhibition of HPTE on androgen production by rat Leydig cells [9,10].

Although our current and previous studies found no effect of 500 nM HPTE on P450scc mRNA levels in luteal and theca cells [18], respectively, a previous study in cultured rat granulosa cells reported that FSH-stimulated P450scc mRNA levels were reduced following exposure to 10 μM HPTE for two days [34]. In this study, lower concentrations of HPTE (0.1 or 1 μM) were without effect

[34]. In addition, the mRNA levels of 3β-hydroxysteroid dehydrogenase and P450 aromatase also were reduced in rat granulosa cells following treatment with 10 μM HPTE [34]. These opposing results could be explained by differences in the concentrations of HPTE used, exposure time or culture conditions used. 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 [11]. This suggests that enzyme activity declined due to reduced protein synthesis. However, in the current study mRNA levels of P450scc in luteal cells were not affected by HPTE, although progesterone levels and P450scc activity declined. Although the decline in progesterone formation in rat luteal 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 or by other possible mechanisms.

Because HPTE acts as an ERα agonist but is antagonistic to ERβ and AR, receptor-linked actions of HPTE in inhibiting progesterone formation are plausible [26]. 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 luteal cells. Some of 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. In the current study and a previous study on follicular cells [18] none of the estrogenic agents including E2 itself, or the xenoestrogenic agents, Bis A or OP, inhibited progesterone formation. When the "pure" estrogen antagonist ICI was added concomitantly with HPTE, it did not alter the inhibition by HPTE of progesterone formation. Similarly, the antiandrogenic agents, MBP, OH-Flu or M2, had no effect on progesterone production by ovarian cells. 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 [9], and the concomitant inclusion of ICI with HPTE did not block the inhibition 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 inclusion of ICI blocked the effects of HPTE [11]. These opposing results could be explained by differences in the cell type (Leydig versus luteal) and age of animals or differences in culture conditions used including the duration and concentrations of the HPTE and ICI exposure. However, in the current study as well as studies with Leydig cells [9,10] including studies by Akingbemi et al. [11], there was a lack of effect of E2 on steroid production by respective cells.

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