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Effects of the common pesticide methoxychlor on ovarian steroidogenesis

Yucel Akgul
West Virginia University

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Effects of the Common Pesticide Methoxychlor on Ovarian Steroidogenesis

Yucel Akgul

Dissertation submitted to the School of Medicine at

West Virginia University

In partial fulfillment of the requirements for the degree of

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in

Physiology and Pharmacology

Eisuke P. Muroso Ph.D., Chair

Robert L. Goodman, Ph.D.

Jorge A. Flores, Ph.D.

Stanley M Hileman, Ph.D.

Ronald J. Millecchia, Ph.D.

Department of Physiology and Pharmacology

Morgantown, WV

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ABSTRACT

Effects of the Common Pesticide Methoxychlor on Ovarian Steroidogenesis

Yucel Akgul

Methoxychlor, an organochlorine pesticide, is released into the environment as a result of its agricultural, home and veterinary use. Following *in vivo* administration, it is quickly metabolized by liver to the proposed active agent, HPTE. Both methoxychlor and HPTE have been shown to exhibit weak estrogenic and antiandrogenic activities, and they are thought to exert their effects through estrogen and androgen receptors. *In vivo* exposure to methoxychlor in laboratory animals causes ovarian atrophy and impaired ovarian steroid production. The effect of methoxychlor and its mechanism(s) of action on ovarian steroidogenesis are not clearly defined. The central hypothesis of these studies is that exposure to HPTE directly inhibits ovarian steroid biosynthesis, which is able to cause an imbalance in circulating progesterone and estradiol levels. **The specific aims of the project are: (1) To pinpoint the potential effect of HPTE on ovarian steroidogenesis, (2) To examine the possible mechanism(s) of action of HPTE on ovarian steroidogenesis, (3) To evaluate the potential effect of HPTE on ovarian luteal steroidogenesis, (4) To determine whether *in vivo* exposure to methoxychlor lowers serum progesterone or estradiol levels and/or inhibits *ex vivo* ovarian steroidogenesis.** Results obtained from the *in vitro* studies demonstrated that HPTE directly inhibits progesterone formation by PMSG-primed rat ovarian theca, granulosa and luteal cells, and this decline in progesterone corresponded to a decline in P450 cholesterol side-chain cleavage activity, which converts cholesterol to pregnenolone in the pathway of progesterone and estrogen biosynthesis. In addition, the percentage decrease in progesterone production was greater than the declines in estradiol and androgen formation suggesting that HPTE may increase the estrogen/progesterone ratio. Further, this effect of HPTE action could be observed at the environmentally relevant concentrations of 10-50 nM and does not appear to be mediated through ER or AR signaling pathways. Consistent with the HPTE action in the *in vitro* studies, *in vivo* exposure to methoxychlor lowers circulating progesterone levels in the immature rats not treated with gonadotropins; however, the exposure of animals to gonadotropins leads to an *ex vivo* increase in progesterone production by isolated ovarian theca cells and no change in circulating progesterone levels. This suggests that the pattern of active steroids secreted *ex vivo* by ovarian cells in response to methoxychlor exposure differs from that observed following *in vitro* exposure to methoxychlor/HPTE.

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GLOSSARY

22(OH)-Cholesterol	22(R)-Hydroxycholesterol
3 β -HSD	3 β -Hydroxysteroid Dehydrogenase
ADN	Adrenodoxin
ADR	Adrenodoxin Reductase
Androstenedione	4-Androsten-3,17-Dione
AR	Androgen Receptor
CL	Corpus Luteum
CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNase I	Deoxyribonuclease I
EDC	Endocrine-Disrupting Chemical
ER	Estrogen Receptor
ER α	Estrogen Receptor α
ER β	Estrogen Receptor β
Estradiol	17 β -Estradiol
Flutamide	4-Hydroxyflutamide
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC	Granulosa Cells
hCG	Human Chorionic Gonadotropin
HDL	High-Density Lipoprotein
HEPES	<i>N</i> -2-Hydroxyethylpiperazine- <i>N'</i> -2-Ethane Sulfonic Acid
HepG2	Human Hepatoma Cells
HPTE	2,2-Bis-(<i>p</i> -Hydroxyphenyl)-1,1,1-Trichloroethane
HSD	Hydroxysteroid Dehydrogenases
HSL	Hormone Sensitive Lipase

ICI	ICI 182,780
LDL	Low-Density Lipoprotein
LH	Luteinizing Hormone
Med 199	Medium 199
MC	Methoxychlor
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Octylphenol	4-Tert-Octylphenol
P450arom	Aromatase, CYP19
P450c17	17 α -Hydroxylase/17,20-Lyase or CYP17
P450scc	P450 Cholesterol Side-Chain Cleavage or CYP11A1
PBR	Peripheral-Type Benzodiazepine Receptor
PBS	Phosphate-Buffered Saline
PCB	Polychlorinated Biphenyl
PCOS	Polycystic Ovarian Syndrome
PKA	Protein Kinase A
PMSG	Pregnant Mare Serum Gonadotropin
PR	Progesterone Receptor
Pregnenolone	5-Pregnen-3 β -ol-20-One
Progesterone	4-Pregnen-3,20-Dione
RIA	Radioimmunassay
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SF-1	Steroidogenic Factor-1
SRB1	Scavenger Receptor B1
StAR	Steroidogenic Acute Regulatory Protein
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>P</i> -Dioxin,
TI	Theca-Interstitial

LITERATURE REVIEW

There are synthetic and naturally occurring compounds that have the potential to mimic and/or interfere with normal functioning of the endocrine system that regulates homeostasis, reproduction, development and behavior [1]. These endocrine-disrupting chemicals (EDCs) are capable of exerting estrogenic, antiestrogenic, androgenic, antiandrogenic or thyroid hormone receptor-induced responses [2]. Many of these EDCs are used as pesticides (e.g., dichlorodiphenyltrichloroethane, DDT; methoxychlor; and vinclozolin), surfactants (e.g., octylphenol and bisphenol A), plasticizers (phthalates), or industrial compounds (e.g., polychlorinated biphenyl, PCB; and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD). Because of the widespread presence of these chemicals in daily life, humans are exposed, starting at conception, to various combinations of them. In fact, these compounds have been detected at low concentrations in almost all human samples including urine, blood, semen, ovarian follicular fluid, amniotic fluid and breast milk (reviewed in [3-5]). Recent articles have proposed that EDCs are involved in the etiology of many reproductive problems, including impaired sperm quality, ovarian failure, polycystic ovarian syndrome (PCOS), and endometriosis, as well as premature puberty and menopause [6-8]. It has been reported that these reproductive disorders have progressively increased over the past 4-5 decades along with the infertility rate in the US [9,10]. However, the casual connection between exposure to potential EDCs and the increased prevalence in reproductive disorders is controversial and not fully accepted [4,6]. Growing evidence from wildlife, epidemiological and

laboratory studies suggest that exposure to various chemicals, starting in utero, could adversely affect adult reproductive health.

In wildlife, adverse reproductive effects including decreased fertility, increased reproductive tract abnormalities, and feminization of male and masculinization of female characteristics were reported in birds, fish and mammals in areas contaminated with EDCs (reviewed in [11]). In farmers or people who live near farms, exposure to pesticides was linked to reduced fertility, sperm damage, menstrual alterations and increased time to pregnancy and increased spontaneous miscarriage rates (reviewed in [12]). In humans, experience with the pharmaceutical diethylstilbestrol (DES) demonstrated that prenatal exposure to a synthetic estrogen could adversely affect reproductive physiology and impair fertility later in life. In utero exposure to DES, prescribed to reduce miscarriage and premature labor, resulted in reproductive abnormalities later in life including increased risk of vaginal adenocarcinomas at puberty and development of breast cancer [13,14]. Supporting these adverse reproductive effects of environmental and occupational levels of EDCs, *in vivo* exposure to relatively higher doses of EDCs in laboratory animals has resulted in similar adverse reproductive outcomes including decreased fertility, precocious puberty, altered estrous cycle expression and gonadal atrophy in rodents and monkeys (reviewed in [15-17]).

One model endocrine disrupter that has the potential to adversely affect reproduction is the pesticide methoxychlor (MC). This dissertation focuses on the effects of MC on ovarian steroidogenesis. The following section will provide an overview of the

metabolism of MC and the potential adverse reproductive effects of MC and its proposed active metabolite, HPTE. This review will also discuss ovarian steroid hormone synthesis and how biosynthesis is regulated..

Methoxychlor

MC is an organochlorine insecticide that is effective against a wide variety of insects. It is released into the environment as a result of its application on fruits, vegetables, forage crops, livestock and pets [18]. MC dissolves poorly in water and can persist in soil for more than a year after its application. It is degraded to dechlorinated, dehydrochlorinated and demethylated products primarily by microorganisms in the soil or water, and this is affected by the level of aeration of the soil [18]. People are exposed to MC and/or its metabolites through inhalation and skin contact during its application, as well as by ingestion of contaminated food and water [18]. Low levels of MC (ranging from 1-4 µg/kg) have been detected in dairy products, grains, fruits and vegetables. Higher levels (10-120 µg/kg) have been infrequently reported in fish [18].

Following *in vivo* administration, MC is rapidly eliminated from the body and does not bioaccumulate in the fat tissue even though it is highly lipophilic (100,000 times higher concentration in the lipid than aqueous phase) [18,19]. MC is converted to demethylated and hydroxylated metabolites mainly by liver cytochrome P450 (CYP) enzymes in both rodents and humans (Figure 1) [19-21]. There is evidence also for peripheral metabolism of MC. For example, the ovarian surface epithelium expresses P450 enzymes that are capable of metabolizing MC [22]. Mechanisms of action of each of these metabolites are

believed to differ; however, among its metabolites, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is known to bind to the estrogen receptor (ER) with a higher affinity than MC itself [23].

In vivo exposure to MC has been reported to cause embryonic toxicity, precocious puberty, decreased fertility and declines in gonadal weight and serum gonadal steroid levels in both male and female rodents [24-27]. In female rats, chronic exposure to MC resulted in acceleration of vaginal opening, disruption of the estrous cycle, reduction in pregnancy rate and a reduction in the number of live pups delivered [28-30]. Further, MC administration increased uterine weight [31] but caused ovarian atrophy [32-34] and a dose-dependent decline in the number of follicles and steroid output [29,34]. Some of these effects were attributed to MC action within the hypothalamic-pituitary axis based on the observations that gonadotropin secretion was altered [24,30]. However, other studies have disputed impaired gonadotropin secretion by MC exposure [30,35], and recent *in vitro* studies suggested direct effects of MC on the gonads [36-39].

Lowest-Observed-Adverse-Effect Levels of Methoxychlor

The most sensitive responses to MC has been seen in female animals, and it appears to reduce ovulation rate and to cause ovarian atrophy in 4-month old gonadotropin-primed adult mice following neonatal i.p. exposure to 0.1-0.5 mg/kg/day MC [32]. Another *in vivo* study reported delayed vaginal opening and decreased ovary weight on postnatal day 46 in female rats following exposure to 5 mg/kg/day of MC (the lowest dose tested) during prenatal day 7 to postnatal day 42 [24]. Although MC doses in these *in vivo* studies are higher than environmentally relevant levels, *in vitro* studies suggested that

MC might alter reproduction at more relevant concentrations. For example, recent studies reported that exposure to 10-100 µg/ml MC in cultured mouse ovarian follicles compromised follicle growth and 100 µg/ml of MC caused antral follicle atresia [36,39]. Furthermore, 100 nM of the MC metabolite HPTE inhibited the production of androgens by cultured rat Leydig cells [37].

Mechanism of Action

In vivo, MC is estrogenic, although its binding affinity for estrogen receptor α (ER α) is ten thousand times less than 17 β -estradiol (estradiol) [40]. MC mimics estradiol action in the female rodent reproductive tract, causing an uterotrophic response in ovariectomized rats [41,42]. In female rodents, exposure to MC caused the proliferation of ovarian surface epithelium through estrogen receptor (ER)-linked mechanisms [43]. Molecular studies revealed that MC and estradiol regulate the activity of many of the same uterine proteins in ovariectomized rodents, including ER [44] and epidermal growth factor receptor [45]. However, estradiol and MC do not exhibit additive or synergistic effects in the reproductive tract of adult ovariectomized mice. For example, combining MC with estradiol suggested some antagonistic effects of MC, as reproductive tract weight changes and induction of uterine albumin levels following treatment with estradiol was blocked by co-administration of MC [46]. Neonatal exposure of mice to lower doses (~6.8 mg/kg or ~13.5 mg/kg) of MC led to a delay in the normal reproductive aging process of ovaries (which is accelerated by estradiol administration) at six months of age [30].

In addition to estrogenic and antiestrogenic properties of MC, recent studies suggested that MC could act through other mechanisms. Ghosh et al. demonstrated that MC induced an increase in mRNA levels of two estrogen-responsive genes, lactoferrin and glucose-6-phosphate dehydrogenase, in ovariectomized ER α knockout mice that could not be blocked by administration of the pure antiestrogen ICI 182,780 [47]. Moreover, it has been shown that MC induced atresia of antral follicles by the activation of Bcl-2- and Bax-mediated signal transduction [39] and oxidative stress pathways [33].

Proposed Active Metabolite HPTE

It is generally acknowledged that the physiological effects of *in vivo* MC treatment are mediated mainly by the metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) [48]. Both MC and HPTE have weak estrogenic and antiandrogenic activities [49,50]. However, HPTE binds with higher affinity to the ER [48] and is a more potent androgen receptor (AR) blocker [49]. In the uterus, which expresses predominantly ER α , it was shown that HPTE and estradiol acted similarly to regulate most gene families including gonadotropin-releasing hormone [51], uterine insulin-like growth factor 1 [52], the progesterone receptor (PR), ER α and AR [53]. In contrast, the induction of ovarian cathepsin B by estradiol was reversed after co-treatment with HPTE, and ER β expression was induced similarly by HPTE and an AR blocker hydroxyflutamide (flutamide) but not by estradiol [53]. In human hepatoma cells (HepG2), HPTE acted as an ER α agonist; however, it was antagonistic to ER β and AR [54]. Although it was generally thought that HPTE exerts its effect through receptor-mediated pathways, some actions of HPTE may not be mediated through the ER or AR. For example, Muroño et al. demonstrated that the

concomitant addition of the “pure” antiestrogen, ICI 182,780 (ICI), did not block the inhibition of HPTE on testosterone formation and the exposure to the antiandrogenic hydroxyflutamide or vinclozolin alone did not mimic the effect of HPTE on androgen production by cultured fetal Leydig cells from neonatal male rats [38].

Effects of MC on Ovarian Steroidogenesis

Limited data is available on the effects of MC on ovarian steroidogenesis. In one study where MC (0, 100 - 500 mg/kg) was administered by gavage to adult female rats on days 1-8 of pregnancy, serum progesterone levels declined beginning at the 100 mg/kg dose [26] (under these conditions, most of the progesterone would have originated from luteal tissue). In a follow-up study, adult female rats were exposed daily to *in vivo* MC (0, 25-500 mg/kg) during the first 8 days of pregnancy, and serum and the *ex vivo* production of progesterone and estradiol by ovarian cells was determined [29]. Similar to the initial study, serum progesterone levels declined starting at the 50 mg/kg dose, then levels were progressively lower at higher doses of MC; however, serum estradiol levels were unaffected by exposure to MC. When minced ovarian tissue from these animals was incubated without or with hCG for 1 h, progesterone production in the tissue exposed *in vivo* to MC, was no different than control; however, both basal and hCG-stimulated testosterone and estradiol formation from these same ovarian tissue declined starting at the 200 mg/kg dose of MC [29]. Another study with young mature female mice demonstrated that the daily i.p. administration of MC for 20 days had no effect on serum estradiol levels even though atresia of antral follicles increased in relation to the dose of MC [36]. *In vitro* studies with ovarian granulosa cells and granulosa cell lines reported that progesterone production was inhibited by exposure to MC or HPTE [55-57];

suggesting that ovarian steroid-secreting cells could be directly affected by both MC and HPTE. In adult male rats, *in vivo* exposure to MC resulted in a reduction in serum testosterone levels and a decline in the *ex vivo* Leydig cell testosterone formation and P450 cholesterol side-chain cleavage (P450_{scc}) activity [58], supporting *in vitro* studies where HPTE directly inhibited testosterone formation by cultured rat Leydig cells [37,38].

Ovarian Steroidogenesis

Steroid hormone production by the ovary is essential for oocyte and follicle development as well as for the initiation and maintenance of pregnancy in mammals [59]. The ovary produces progestins, androgens and estrogens in a sequential manner, with each serving as substrate for the subsequent steroid, and the levels secreted vary with the stage of follicle development [60]. The preovulatory follicle (multiple follicles in rodents) secretes mainly estrogens (estradiol and estrone) along with androgens (dehydroepiandrostenodione, androstenedione and testosterone), which reach higher levels shortly before ovulation [61]. The corpus luteum primarily produces progesterone along with estrogens [61]. Timely production of the ovarian steroids and the subsequent activation of local steroid-mediated signaling is important for development of the oocyte and follicles [62]. For example, mice with reduced expression of ERs or lacking AR are reported to have abnormal follicle growth, ovarian failure and altered oocyte maturation [63-66]. Mice lacking PRs do not ovulate in response to gonadotropins [67]. In addition, excess ovarian steroid secretion can lead to significant ovarian pathology, as increased ovarian steroid signaling leads to polycystic ovarian syndrome (PCOS), which is

characterized by excess androgen production, abnormal follicle growth and anovulation in women [62].

Ovarian Follicle

The main steroid-secreting ovarian cells (theca and granulosa) are organized in a structure called the follicle, which represents the basic functional unit of the ovary with respect to oocyte development and steroid production [61]. Follicles in varying developmental stages (primordial, primary, preantral, antral, preovulatory and corpus luteum) are embedded in connective tissue of the ovarian cortex [61]. The majority of these follicles are primordial (small resting follicles that contains an immature oocyte and a single layer of flattened granulosa cells enclosed by a basal lamina), and they do not have theca cells associated with them [68]. Only after development has proceeded to the preantral stage when a second layer of cuboidal granulosa cells begin to appear, do the first theca cells begin to differentiate from the interstitial cells of the ovarian stroma [68]. Prior to puberty, resting follicles can enter the growing pool, and they can progress to the antral stage at which point they inevitably undergo atresia [59]. Although gonadotropins stimulate growth and differentiation of preantral follicles, follicles can develop up to the antral stage in the absence of gonadotropins [59]. Starting at puberty, a cohort of antral follicles escapes apoptosis due to follicle stimulating hormone (FSH) action [59] and develops into mature follicles destined to either regress or form the corpus luteum following ovulation (figure 2). As the selected follicle grows, its antrum is engorged with follicular fluid, both theca and granulosa cells proliferate, and they produce increasing levels of steroid hormones. In theca cells, luteinizing hormone (LH) receptors and the

enzyme 17 α -hydroxylase/17,20-lyase (P450c17), which converts pregnenolone and progesterone to dehydroepiandrosterone (DHEA) and androstenedione, respectively, are expressed primarily. In granulosa cells, mainly FSH receptors and aromatase (P450arom), which converts androgens to estrogens, are expressed [61].

In the preovulatory follicle shortly before ovulation, both the granulosa and theca cells are stratified in a distinct manner [61]: (1) Cumulus granulosa cells, which lack steroidogenic enzymes and express low levels of LH receptors, surround the mature oocyte and are extruded with the oocyte at ovulation. (2) The remaining granulosa cells in the follicle (the layers between basement membrane and the antrum filled with steroid rich follicular fluid) are named membrana granulosa, and they have high levels of LH receptors and steroidogenic enzymes to produce progesterone from cholesterol and estrogens from androgens. However, the granulosa cells that abut the basement membrane seem to be steroidogenically and metabolically more active than those close to the antral cavity. (3) Theca-interstitial cells are identified as theca interna, the stromal cell layer adjacent to the basal lamina, and theca externa, a less defined layer of stromal cells that make up the outermost layer of the follicle. Unlike granulosa cells, the theca compartment is highly vascularized [59]. In the atretic follicles, both granulosa and theca cells disappear; however, theca cells may persist and remain in the ovarian stroma as nests of secondary interstitial cells for a while. These cells retain the ultrastructural characteristics of theca cells and continue to respond to LH by secreting steroid hormones [68].

Following ovulation with the release of the oocyte-cumulus complex, the remaining theca and granulosa cells undergo structural and genomic transformation to become a progesterone-producing endocrine gland, the corpus luteum [69]. Some of the major alterations during luteinization of granulosa and theca cells include: (1) increased expression of key proteins involved in the uptake, synthesis and transport of cholesterol, and in the processing of cholesterol to progesterone, androgens and estrogens, (2) changed expression of various receptors that allow luteal cells to respond to a new set of hormones, such as the upregulation of prolactin receptors, silencing of FSH receptors, and a shift to the predominant expression of $ER\alpha$ from $ER\beta$ (reviewed in [69]). The formation of the corpus luteum also involves the breakdown of the follicular basal membrane which allows migration of endothelial cells, fibroblasts and theca cells into the previously avascular granulosa cells [69].

Once luteinization takes place, granulosa and theca cells undergo extensive hypertrophy, and these cells differentiate into large and small steroidogenic luteal cells, respectively, with the large cells producing 2- to 40-fold more progesterone than the small cells [69]. In contrast to the primate corpus luteum, in rodents, small and large luteal cells are intermingled within the corpus luteum, and they do not appear to differentiate from the theca and granulosa cells, respectively. Furthermore, in rodents both cell types express proteins that allow cholesterol conversion into estrogens [61]. Luteal cells produce steroid hormones until luteolysis (luteal cells lose their steroidogenic capacity and are eventually resorbed) unless steroidogenesis is maintained by human chorionic gonadotropin (hCG) [68].

Regulation of Steroidogenesis

The secretion of ovarian steroid hormones is tightly regulated by the neuroendocrine system (hypothalamic-pituitary-gonadal axis) and is locally controlled by intraovarian factors including steroids, glycopeptides and cytokines [70]. This complex process involves several different signaling pathways and multiple types of cells [62].

Steroidogenesis in most mammals appears to mainly depend on the activation of the cAMP/protein kinase A (PKA) pathway by pituitary-derived LH and FSH in theca and granulosa cells, respectively (the so called two-cell, two-gonadotropin model). In this model, androgens produced by the LH-stimulated theca cells are converted to estrogens by the FSH-stimulated granulosa cells (figure 3) [61]. LH is also a key regulator of steroidogenesis in the luteal cells in humans [61]; however, in rodents, LH, prolactin and estradiol appears to be critical luteotrophic hormones, and the cAMP/PKA pathway is not the primary route utilized by luteal cells [71].

Steroidogenic cells do not store significant quantities of steroid hormones; hence, steroid secretion is directly related to steroid synthesis, which is regulated both acutely and chronically. Activation of the cAMP/PKA pathway causes a rapid increase in the delivery of cholesterol into the inner mitochondrial membrane where the first step(s) of steroidogenesis occurs. This involves activation of cholesterol esterase and carrier proteins (e.g, steroidogenic acute regulatory protein, StAR, see below) within minutes to a few hours [72]. Acute regulation of steroidogenesis involves also increasing the catalytic rate of steroidogenic enzymes, which is regulated by the associated electron transfer process [73]. Chronic stimulation of cAMP/PKA signaling, which takes several hours, is through the phosphorylation of a number of transcription factors in the nucleus

to stimulate the expression of genes involved in regulating steroidogenic enzyme levels [72]. Among these transcription factors, steroidogenic factor-1 (SF-1, a member of the orphan nuclear receptor family) appears to act as a master switch to initiate transcription of a series of steroidogenic genes by binding to their response elements on the appropriate promoter regions of the genes [61]. These genes encode key proteins involved in the uptake, synthesis, transport and in the processing of cholesterol in each cell type [61]. These proteins include: (1) StAR, P450 side-chain cleavage (P450scc) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in both theca and granulosa cells, which are capable of making pregnenolone and progesterone from cholesterol, (2) P450c17, which catalyzes androgen synthesis from pregnenolone and progesterone in theca cells, and (3) P450arom that converts androgens to estrogens in granulosa cells [61].

Besides transcriptional regulation of these enzymes, steroidogenic pathways are regulated by electron-donation and the redox state of cells [73]. Two categories of enzymes are involved in steroid synthesis (figure 4), including cytochrome P450 (CYP) and hydroxysteroid dehydrogenases (HSD). There are two types of cytochrome P450s according to their intracellular locations, mitochondrial type I and microsomal type II. P450scc (product of the CYP11A1 gene) is loosely associated with the inner mitochondrial membrane (cytochrome P450 type I), whereas the remaining P450s (e.g., P450c17 and P450arom) are anchored on the inner membrane of the endoplasmic reticulum (type II) [72]. Generally, cytochrome P450s function as monooxygenases utilizing electrons donated from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to catalyze the hydroxylation and cleavage of substrates [72]. The HSDs,

which include 3 β -HSD and 17 β -HSD, are involved in the reduction and oxidation of steroid hormones. They require NAD⁺/NADP⁺ as electron acceptors, while their reduced forms serve as donors of reducing equivalents [74].

Steroid Hormone Biosynthesis

Transport of Cholesterol into Mitochondria

The biosynthesis of sex steroids starts from cholesterol, which is a precursor of all steroids [75]. Cholesterol can be synthesized *de novo* from acetyl coenzyme A or hydrolyzed from stored cholesterol esters in most steroidogenic cells. However, the major mechanisms for providing cholesterol are either the endocytosis of cholesterol rich low-density lipoprotein (LDL) via LDL receptor or the selective uptake of cholesterol esters from high-density lipoprotein (HDL) via scavenger receptor B1 (SRB1), with the relative contribution of these two types of lipoproteins varying among species [75]. Once liberated as free cholesterol, it is immediately utilized, or it can be stored as cholesterol esters in lipid droplets [75] (Fig. 2). Because cholesterol is poorly soluble in water, it must be transported by carrier proteins to the inner mitochondrial membrane where P450_{scc} is located to be used as a steroid hormone precursor. Among these proteins, StarD4, which is structurally related to the StAR, has been proposed to play a major role in the cytosolic movement of free cholesterol to the outer mitochondrial membrane in steroidogenic cells [75]. The movement of cholesterol from the outer to inner mitochondrial membranes is facilitated primarily by StAR, the first rate-limiting and regulated step in ovarian steroid biosynthesis [75]. The crucial role of StAR in

steroidogenesis has been illustrated by the targeted disruption of the StAR gene, which results in severely impaired gonadal and adrenal steroid synthesis [76]. Peripheral-type benzodiazepine receptor (PBR) and hormone sensitive lipase (HSL) were also shown to interact with StAR to facilitate cholesterol translocation from the outer to the inner mitochondrial membranes through the aqueous intermembrane space [77,78] (figure 5).

P450 Cholesterol Side-chain Cleavage System

The net steroidogenic capacity of a cell is determined by the expression of mitochondrial P450_{scc}, which converts cholesterol to pregnenolone in three sequential steps, 20 α -hydroxylation, 22-hydroxylation and C20,22-bond scission, at a single active site [79]. P450_{scc} must receive electrons from NADPH through the intermediacy of two electron transfer proteins, adrenodoxin reductase (ADR, a flavoprotein) and adrenodoxin (ADN, an iron/sulfur protein) [80]. In this reaction, the side-chain of the substrate cholesterol gets cleaved off to yield pregnenolone and isocaproic aldehyde at the expense of 3 molecules of NADPH and 3 molecules of oxygen (figure 6) [81].

P450_{c17} and 3 β -Hydroxysteroid Dehydrogenase

Following synthesis of pregnenolone by P450_{scc} in the mitochondria, pregnenolone then moves to the endoplasmic reticulum where it is converted either to 17-hydroxypregnenolone by P450_{c17} [82] or to progesterone by 3 β -HSD [83]. Thus, the steroidogenic pathway bifurcates into a Δ^5 -hydroxysteroid pathway (starting with pregnenolone) and a Δ^4 -ketosteroid pathway (starting with progesterone). Although the

same enzymes use different substrates along these parallel pathways, both pathways converge to produce androstenedione (figure 4).

As mentioned above, the StAR/P450_{scc} system is rate-limiting and the quantitative regulator of steroidogenesis. In contrast, P450_{c17} is the qualitative regulator, directing pregnenolone toward its final metabolic pathway, which is androstenedione in the theca cell [61]. Androstenedione can then be converted to testosterone by 17 β -HSD.

Aromatase and 17 β -HSD

Local and circulating levels of estrogen are controlled by P450_{aromatase}, the product of the CYP19 gene [84]. The heme protein is responsible for binding of the androgenic steroid substrate and catalyzing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens [84]. Tissue-specific promoters and alternative splicing control expression of aromatase in multiple tissues including ovary, bone, adipose tissue, brain and various fetal tissues [84].

17 β -HSD enzymes catalyze the final step in the biosynthesis of active gonadal steroid hormones, estradiol and testosterone. Among the many different forms of 17 β -HSDs, 3 forms participate in the final step of biosynthesis of active steroid hormones in gonads, types I, III and VII [74]. The type I form is responsible for follicular phase estradiol production by granulosa cells and is primarily induced by FSH acting via the cAMP/PKA pathway, whereas following silencing of FSH action after ovulation, type VII is responsible for estradiol formation by the corpus luteum (review in [74]).

Steroid Hormone Metabolism

The ovary produces mainly two biologically active ovarian steroids, estradiol and progesterone, along with less active or inactive steroid hormones such as pregnenolone and androstenedione [61]. Circulating levels of each of these hormones is regulated by the rate of ovarian secretion as well as peripheral metabolism by the liver, adipose tissue, skin, uterus and ovary. For example, in the ovary and uterus, progesterone can be 'inactivated' by its conversion to 20 α -hydroxyprogesterone, which is a less active hormone, by 20 α -hydroxysteroid dehydrogenase [85]. Another pathway for metabolism in rodents is by 6 β - or 16 α - hydroxylation of progesterone in the liver [86,87], which are mediated by CYP3A and CYP2B enzymes, respectively [88].

Figures

Figure 1. Proposed metabolic pathway of methoxychlor catalyzed by human P450s

Methoxychlor is first *O*-demethylated to mono-OH-M, which is then either *ortho*-hydroxylated to form catechol-M or is further *O*-demethylated to yield bis-OH-M. The final product tris-OH-M is then generated from bis-OH-M via *ortho*-hydroxylation and from catechol-M by *O*-demethylation.

This diagram is adapted from [89]

Figure 2. Ovarian morphology and ultrastructure of theca and granulosa cells

(A) primordial follicle; (B) primary follicle; (C) secondary follicle; (D) small antral follicle; (E) components of antral follicles; (F) ultrastructure of a theca cell. N, nucleus; M, mitochondria with vesicular cristae; SEM, smooth endoplasmic reticulum; L, lipid vesicles containing cholesteryl esters; *, connective tissue containing collagen fibers.

This diagram is adapted from [68]

Figure 3. The two-cell, two-gonadotropin concept of follicle estrogen biosynthesis

This diagram is adapted from [68]

Figure 4. Steroidogenic pathway in the ovary

This diagram is adapted from [61]

Figure 5. Principal features of the cellular cholesterol economy

This diagram is adapted from [75]

Figure 6. Schematic overview of the CYP11A1 electron-providing chain

This diagram is adapted from [81]

Project Goals and Aims

The effect of MC and its mechanism(s) of action on ovarian steroidogenesis are not clearly defined. This project was designed to determine whether MC is able to alter ovarian steroidogenesis via its proposed active metabolite HPTE. The central hypothesis of this work is that exposure to HPTE directly inhibits ovarian steroid biosynthesis, which is able to cause an imbalance in circulating progesterone and estradiol levels. It is well established that chemicals that cause ovarian steroid imbalance could have a significant effect on fertility, menstrual and estrous cycle, and timing of puberty or menopause. The specific aims of the project are: (1) To pinpoint the potential effect of HPTE on ovarian steroidogenesis, (2) To examine the possible mechanism(s) of action of HPTE on ovarian steroidogenesis, (3) To evaluate the potential effect of HPTE on ovarian luteal steroidogenesis, (4) To determine whether *in vivo* exposure to MC lowers serum progesterone or estradiol levels and/or inhibits *ex vivo* ovarian steroidogenesis.

**STUDY 1: AN ACTIVE METABOLITE OF THE PESTICIDE
METHOXYCHLOR, HPTE, REDUCES PROGESTERONE,
ANDROGEN AND ESTRADIOL PRODUCTION BY RAT
OVARIAN CELLS BY INHIBITING THE CHOLESTEROL
SIDE-CHAIN CLEAVAGE STEP**

Abstract

Previous *in vivo* studies in female rodents demonstrated decreased progesterone production and no change in serum estrogen levels following exposure to methoxychlor. It is not established how MC exerts its effect on ovarian steroidogenesis. The current *in vitro* studies examined the effects of HPTE on androgen and progesterone production by cultured ovarian granulosa cells and theca interstitial cells from immature rats treated with 20 I.U. of pregnant mare serum gonadotropin. These studies have demonstrated that HPTE progressively inhibits progesterone formation by granulosa and theca interstitial cells and androgen formation by TI cells. These effects were observed at 50 nM HPTE, and the main site of action appeared to be localized at the P450 cholesterol side-chain cleavage step, which converts cholesterol to pregnenolone in the pathway of progesterone and estrogen biosynthesis. In addition, the percent decrease in progesterone production was greater than the decline in estradiol and androgen levels suggesting that HPTE may increase the ovarian estrogen/progesterone ratio. These studies also provide evidence that HPTE does not exert its effect on ovarian steroidogenic cells by blocking cholesterol transport into mitochondria or by inhibiting microsomal enzymes involved in the conversion of pregnenolone to estrogen.

1. Introduction

Environmental or occupational exposure to endocrine-disrupting chemicals (EDCs) have 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 pesticide methoxychlor (MC), which is released into the environment as a result of its applications to agricultural crops, livestock, animal feed, stored grain, home gardens and pets [3]. *In vivo*, MC exposure alters reproductive and ovarian function in laboratory rodents. Some of the reported adverse effects include embryonic toxicity [4], precocious puberty [5], decreased fertility [6], ovarian atrophy [7-9] and a dose-dependent decline in the number of follicles [10] and steroid production [11]. 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) [12]. Although the conversion of MC to HPTE occurs primarily in the liver, there is evidence for peripheral metabolism. For example, the ovarian surface epithelium expresses cytochrome P450 (CYP) enzymes that are capable of metabolizing MC [13]. Both MC and HPTE have weak estrogenic and antiandrogenic activities [14-16]. However, HPTE binds with higher affinity to the estrogen receptor (ER) [12], and it is a more potent androgen receptor (AR) blocker than MC [16]. In human hepatoma cells (HepG2), HPTE acted as an estrogen receptor α (ER α) agonist, but it acted as an antagonist to estrogen receptor β (ER β) and the androgen receptor (AR) [17].

HPTE and its parent compound MC are reported to induce proliferation of the ovarian surface epithelium [18] and to inhibit progesterone secretion in cultured granulosa cells

(GC) [19,20]. These limited studies do not address the question of how MC or HPTE exert their effects on ovarian steroidogenesis. However, studies on male gonads suggested potential mechanisms of action of HPTE on steroidogenesis [21-24]. Murono et al. reported that HPTE inhibited both basal and hCG-stimulated testosterone formation by Leydig cells in a dose-dependent manner in both neonatal and young adult rats [22,23]. In these studies, significant declines in testosterone were observed at about 100 nM HPTE, and this action was associated with a direct inhibition of P450 cholesterol side-chain cleavage (P450_{scc}) activity. Up to now, it is not known whether HPTE has any effect on steroid formation by ovarian theca cells. The current *in vitro* studies examined the effects of HPTE on androgen and progesterone production by cultured ovarian theca-interstitial (TI) cells and progesterone and 17 β -estradiol formation by cultured GC from ovaries of immature rats.

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., Scottsdale, 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 Alpha-dri (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 Purina rat chow (R-M-H 3500 with 5% fat content) and tap water ad libitum. Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All animal protocols were reviewed and approved by the local Animal Care and Use Committee.

2.2. Reagents

Collagenase (Sigma Blend Type L), penicillin G, streptomycin sulfate, deoxyribonuclease I (DNase I), 22(*R*)-hydroxycholesterol, 5-pregnen-3 β -ol-20-one (pregnenolone), 4-pregnen-3,20-dione (progesterone), 4-androsten-3,17-dione (androstenedione), dimethylsulfoxide (DMSO), and pregnant mare serum gonadotropin (PMSG) were purchased from Sigma, St. Louis, MO, USA. Ecolite (liquid scintillation fluid) was from ICN Pharmaceuticals Inc., Costa Mesa, CA, 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. [2,3,6,7-³H(N)]-Testosterone was from Perkin-Elmer Life Sciences, Boston, MA, USA. Unlabeled testosterone and 17 β -estradiol (estradiol) were from Steraloids, Wilton, NH, USA. 2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, 99% pure) was from Cedra Corp., Austin, TX, USA. RIA kits to quantitate progesterone, androstenedione and estradiol were purchased from Diagnostic Products Corporation, Los Angeles, CA.

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/0.1ml). Animals were treated between 0800 and 1000 h each day. Approximately 48 h following PMSG injection, animals were sacrificed by intra-peritoneal pentobarbital injection. For each experiment, 3-4 animals were sacrificed to yield a single pool of cells for culture in a given study.

2.4. Isolation and Incubation of Whole Follicle, Theca Interstitial and Granulosa Cells

Animals were 25-27 days of age when sacrificed. The ovaries were immediately removed, rinsed and stripped of its bursa and fat tissue in Med 199. Using fine microsurgery forceps, whole follicles of ~1 mm diameter were teased out and placed in pairs into 24-well Costar culture plates containing 1 ml of a 1:1 mixture of DMEM/F-12 medium and 15 mM HEPES (pH 7.4), 15 mM NaHCO₃, 100 U/ml penicillin G, and

100 µg/ml streptomycin. Follicles were incubated with 10 µM HPTE (added on the day of plating) for 24 h in DMEM / F12 medium at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂. Fresh HPTE (dissolved in DMSO) was prepared for each experiment. Granulosa cells (GC) and theca-interstitial (TI) cells were isolated by slight modifications of the methods described previously [26,27]. Briefly, the ovaries were harvested, rinsed, stripped of its 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 was centrifuged (209 x 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 Med 199 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 50 min. Finally, ice-cold Medium 199 was added to stop further dissociation of the residual tissue, and the cell suspension was centrifuged (209 x g) for 10 min to yield TI cells. The final pellet was then resuspended in DMEM/F-12 medium.

The 3β-hydroxysteroid dehydrogenase (3β-HSD) staining revealed that 15-20 % of TI cells and 60-70 % of GC pellet consisted of steroidogenic cells (theca and granulosa cells respectively). Remaining cells are vascular , stromal, fat etc. In addition the 3β-HSD staining revealed that GC stained with less intensity than TI cells.

GC and TI cells (10⁵/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 NaHCO₃,

100 U/ml penicillin G, and 100 µg/ml streptomycin as described previously [26]. Cells were exposed to HPTE (0, 10, 50, and 100 nM) on the day of plating and cultured for 24 h at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂. Each experiment was repeated at least three times. The HPTE concentrations were selected based on preliminary sensitivity studies, which demonstrated that cultured GC and TI became detached from culture plates at HPTE concentrations above 1 µM. In addition, although plated cells (both control and HPTE treated) were viable for more than 7 days under the culture conditions used, their steroid output declined after about 24 h.

2.5. Measurement of Progesterone, Androstenedione and Estradiol

Progesterone, androstenedione and estradiol were quantitated directly from the collected medium by radioimmunoassay (RIA) as described in the manufacturer's protocol.

2.6. Measurement of Testosterone

Testosterone released into the medium was quantitated directly by specific RIA as described previously [28].

2.7. Statistical Analysis

Data were expressed as the mean $\pm$ 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, Testosterone and Estradiol Production by Whole Follicles

Studies were initiated to determine whether progesterone, testosterone and estradiol formation by ovarian follicles was sensitive to HPTE. As described in Methods, the isolated follicles were placed in pairs into each well of a 24-well culture plate in 1 ml medium. Follicles were cultured with 10 μ M HPTE for 24 h. Statistically significant declines were observed in each of the measured steroids. Progesterone levels declined from 19.72 ± 1.91 ng/ml in control follicles to 8.15 ± 0.94 ng/ml (~41 % of control, Figure 1, Panel A). Testosterone levels declined from 1.02 ± 0.06 ng/ml in control follicles to 0.78 ± 0.06 ng/ml (~76 % of control, Figure 1, Panel B) and estradiol levels declined from 32.67 ± 3.99 ng/ml in control follicles to 21.77 ± 3.11 ng/ml (~67 % of control, Figure 1, Panel C). The percent declines in testosterone and estradiol levels in response to HPTE were similar, but their decrease was less than the fall in progesterone levels.

3.2- Effect of HPTE on Progesterone, Androstenedione and Testosterone Formation by TI Cells

This study was conducted to localize the site(s) within the ovarian follicle where HPTE inhibited steroidogenesis. PMSG-stimulated ovaries were harvested, and TI cells were isolated and plated. Cells were incubated with varying concentrations of HPTE (0, 10, 50 and 100 nM) for 24 h. The progesterone level in the control wells was 19.16 ± 0.78 ng/ml/ 10^5 cells (Figure 2, Panel A). A decline in progesterone was observed with 10 nM

HPTE, which declined progressively to 1.40 ± 0.29 ng/ml/ 10^5 cells (7 % of control) following exposure to 100 nM HPTE. Similarly, androstenedione levels declined from 13.72 ± 1.09 ng/ml/ 10^5 cells in control cells to 5.53 ± 0.52 ng/ml/ 10^5 cells (40 % of control) in cells exposed to 100 nM HPTE (Figure 2, Panel B). Testosterone levels declined from 119.05 ± 10.06 pg/ml/ 10^5 in control cells to 36.99 ± 2.60 pg/ml/ 10^5 cells (31 % of control) in cells exposed to 100 nM HPTE (Figure 2, Panel C). There is no statistical difference between testosterone and androstenedione production with the highest HPTE concentration when they are compared as a percentage decline. This suggested that the decline in the secretion of these androgens after exposure to HPTE followed a similar pattern.

We also evaluated whether HPTE alters progesterone production by GC. Progesterone levels declined from 5.03 ± 0.54 ng/ml/ 10^5 cells in control cells to 0.51 ± 0.07 ng/ml/ 10^5 cells (10 % of control) in cells exposed to 100 nM HPTE for 24 h, indicating the adverse effect of HPTE on GC is similar to its effect on TI cell progesterone formation.

3.3- Localizing the Effect of HPTE On Ovarian Steroidogenic Pathway

3.3.1. Role of HPTE on the delivery of cholesterol to the inner mitochondrial membrane

To determine whether HPTE alters the translocation of cholesterol to the inner mitochondrial membrane, a readily diffusible form of cholesterol, 22(R)-hydroxycholesterol (22(OH)-cholesterol), was used. Since hydroxylated cholesterol does not require transporters to reach the inner mitochondrial membrane [29], supplying this substrate to TI cells and measuring downstream steroid hormone levels namely

progesterone and testosterone, may reveal whether HPTE affects the delivery of cholesterol to the inner mitochondrial membrane where P450 cholesterol side-chain cleavage (P450_{scc}) activity is localized. In the presence of 10 μ M 22(OH)-cholesterol, the progesterone level in the control group was 351.92 ± 11.47 ng/ml/ 10^5 cells (Figure 3, Panel A). A significant decline in the progesterone level was observed following exposure to 50 nM HPTE, which declined further to 121.67 ± 3.51 ng/ml/ 10^5 cells (35 % of control) following exposure to 100 nM HPTE. Testosterone levels declined from 557.05 ± 56.79 pg/ml/ 10^5 cells in the control group to 263.44 ± 21.36 pg/ml/ 10^5 cells (47 % of control, Figure 3, Panel B) in cells treated with 100 nM HPTE. These results imply that cholesterol transport into mitochondria was not affected by HPTE and that an enzymatic step(s) converting cholesterol to downstream steroid products was inhibited.

3.3.2- Effect of HPTE on microsomal enzymes converting pregnenolone to testosterone in cultured TI cells

To test the effect of HPTE on 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity, which converts pregnenolone to progesterone, TI cells were co-cultured with HPTE (0, 10-100 nM) and pregnenolone (10 μ M) as a substrate. Next, the formation of progesterone and testosterone was determined following exposure for 24 h (Figure 4). There was no statistically significant difference either in progesterone production (23.6 ± 1.56 μ g/ml/ 10^5 cells in control, Panel A) or testosterone production (2.28 ± 0.46 ng/ml/ 10^5 cells in control, Panel B) by TI cells following exposure to 10-100 nM HPTE when 10 μ M pregnenolone was provided as substrate. This suggests that 3 β -HSD activity is not affected by HPTE. Also, this pattern of androgen formation suggests that 17 α -

hydroxylase/C17,20-lyase (P450c17) activity, which converts progesterone to 17 α -hydroxyprogesterone and androstenedione, and 17 β -hydroxysteroid dehydrogenase (17 β -HSD) activity, which converts androstenedione to testosterone, are unaffected by HPTE. TI cells were also co-treated with 10 μ M progesterone or androstenedione (10 μ M) as substrates and varying concentrations of HPTE (10-100 nM) to more specifically evaluate the effect of HPTE on P450c17 and 17 β -HSD activities, respectively. The steroid product testosterone was quantitated following 24 h of incubation when either substrate was included in the culture medium. There were no statistically significant differences in testosterone levels between control and HPTE treated groups, suggesting that neither P450c17 nor 17 β -HSD activity is affected by HPTE (Figures 5 and 6).

3.3.3 Effect of HPTE on aromatase activity

For antral follicles to produce estradiol, the coordination between GC and TI cells is required [30]. Therefore, because GC have low P450c17 activity, they are unable to convert progesterone to androgens, while TI cells lack aromatase activity and, therefore, can not synthesize estradiol from androgens (androstenedione and testosterone) [30,31]. For estradiol to be synthesized by antral follicles, the androgens synthesized by TI cells must be transferred to GC where aromatase is located.

To demonstrate whether HPTE affects P450 aromatase activity, which converts testosterone to estradiol, GC were cultured with testosterone (10 μ M) as substrate and varying concentrations of HPTE (10-100 nM). There was no statistically significant

difference in estradiol production (149.02 ± 25.05 pg/ml/ 10^5 cells in control) by GC cells implying P450 aromatase is not affected by HPTE (Figure 7).

Discussion

The current studies have demonstrated that the reported active metabolite of MC, HPTE, progressively inhibits progesterone formation by GC and TI cells, and androgen formation by TI cells from immature rats. These effects could be observed at 50 nM HPTE, and the main site of action appeared to be localized at the P450_{scc} step, which converts cholesterol to pregnenolone in the pathway of progesterone and estrogen biosynthesis. In addition, the percentage decrease in progesterone production was greater than the declines in estradiol and androgen formation suggesting that HPTE may increase the estrogen/progesterone ratio. Because the inclusion of exogenous substrates with HPTE to cultured ovarian cells demonstrated that co-incubation with 22(OH)-cholesterol inhibited steroidogenesis, while the addition of other steroid substrates (pregnenolone, progesterone, androstenedione or testosterone) produced after the P450_{scc} step did not, this suggested that the primary site of HPTE action was at the P450_{scc} step. The results suggested also that neither the transport of cholesterol to the inner mitochondrial membrane (where P450_{scc} is localized) nor the enzymatic steps (3 β -HSD, P450_{c17}, 17 β -HSD or aromatase) involved in the conversion of pregnenolone to androgens or 17 β -estradiol, are inhibited by HPTE. This action of HPTE does not appear to be cell specific since progesterone formation by both GC and TI cells were affected in the current study. A recent study also reported that HPTE lowered FSH-stimulated progesterone formation by isolated GC from immature rats [20]. Besides follicular cells, previous *in vitro* studies on isolated rat Leydig cells demonstrated that basal and hCG-stimulated testosterone formation are decreased by HPTE inhibition of P450_{scc} activity. The parent compound

of HPTE, MC, was also shown to inhibit P450_{scc} activity in isolated mitochondria from the bovine adrenal cortex [32] and progesterone formation by porcine GC [11,21].

The present *in vitro* observations indicate that HPTE specifically inhibits the P450_{scc} step in the pathway of converting cholesterol to androgens and 17 β -estradiol without affecting the other steps in the ovarian steroidogenic pathway. This conclusion was based on the level and pattern of steroid products produced following the co-incubation of cultured GC or TI cells with HPTE and exogenously supplied 22(OH)-cholesterol or various steroid substrates. Because 22(OH)-cholesterol does not require steroidogenic acute regulatory (StAR) protein to be transported to the inner mitochondrial membrane [29] where P450_{scc} is localized, these results suggest that HPTE does not block StAR function in translocating cholesterol to the site of P450_{scc} activity. Our results also support the observations of other studies reporting that HPTE did not affect StAR mRNA levels, the primary transporter of cholesterol into mitochondria in GC and Leydig cells [20,25]. However, measuring mRNA levels reflects only the potential effects of HPTE on StAR levels and not actual levels. The current observations with TI cells confirm the results of a previous study on cultured rat Leydig cells where co-incubation with HPTE and the substrate 22(OH)-cholesterol resulted in the progressive decline in testosterone formation, while HPTE had no effect on testosterone production when pregnenolone, progesterone or androstenedione were added as substrates [24]. In the present studies, the conversion of testosterone to estradiol was not affected by 100 nM HPTE in GC suggesting that HPTE has no effect on aromatase activity. In contrast, Zachow et al reported declines in estradiol formation and P450_{aromatase} mRNA levels

following exposure of rat GC to 10 μ M HPTE [20]. It should be noted that the concentration of HPTE used in this study was three orders of magnitude higher than was used in the current study.

Although there is general agreement that HPTE inhibits the P450scc step, the mechanism of action is not established. Muroño et al showed that HPTE (100 nM) directly inhibited the catalytic activity of P450scc, and that the ER or AR were not involved [23,24]. In contrast, Akingbemi et al reported that the decline in testosterone formation following exposure to 1 μ M HPTE was associated with a decline in steady state P450scc mRNA levels, and they suggested that the effects of HPTE were mediated through the ER pathway [25]. Furthermore, a recent study in cultured rat GC showed that FSH-stimulated mRNA levels of P450scc, 3 β -HSD and P450aromatase were reduced by 10 μ M HPTE indicating that this action may not be specific to P450scc [20]. In this study, a lower concentration of HPTE (1 μ M) as shown to inhibit progesterone formation. Therefore, it is possible that higher concentrations of HPTE alters mRNA levels of multiple steroidogenic enzymes while lower concentrations may be more specific to P450scc activity leading to decreased steroid formation. We observed an increased detachment of cultured GC and TI cells at HPTE concentrations of 1 μ M or higher, suggesting possible toxicity at higher concentrations (data not shown).

Our current *in vitro* studies and other studies indicate that HPTE directly lowers GC and TI progesterone production and this may contribute to the decline in serum levels that was reported when female rodents were exposed *in vivo* to MC [6,12]. It has been shown

in previous studies that MC causes an increase in the number of atretic antral follicles [7,33]. A potential consequence of follicular atresia is a decrease in serum progesterone levels. But, it has not been determined how MC exposure causes follicular atresia, an active and intrinsic process [34,35]. Gonadotropins and estradiol have been reported to prevent apoptosis of GC from antral follicles while androgens induce it [36,37]. However, the role of progesterone in inducing follicular atresia has not been ruled out. Progesterone receptor protein and mRNA were localized to the theca of small preantral and antral follicles and to GC of preovulatory follicles that had been exposed to LH/hCG in the sheep, monkey and rabbit ovaries [38-40]. High levels of progesterone increased atresia of bovine nonovulatory dominant follicles [31], whereas progesterone inhibited apoptosis in cultured rat GC [41]. Therefore, based on our current results with HPTE, it is plausible that the direct inhibition of ovarian progesterone production, or alteration of the delicate balance between progesterone, androgen and estrogen levels locally, may actually be the cause of atresia in antral follicles.

In conclusion, this study suggests that the HPTE specifically inhibits the P450_{scc} step in ovarian steroidogenesis, which may decrease progesterone and estradiol formation in PMSG-primed ovarian cells from immature rats. Furthermore, this work indicates that HPTE may increase the estrogen/progesterone ratio, which may adversely affect normal follicular development. Further studies should examine the mechanism of action of HPTE on ovarian steroidogenesis.

Figures

Figure 1. 1. Effect of HPTE on whole follicle progesterone, testosterone and estradiol formation

Ovarian follicles of around 1 mm in diameter were placed in pairs into each well of 24-well culture plate and exposed to HPTE (10 μM) for 24 h. HPTE was dissolved in DMSO, and all treatment groups contained 0.1 % of the vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of 12 pairs of follicles from a single experiment. (a, b), $P < 0.050$ and $P < 0.001$, respectively, when compared with the appropriate control group

Figure 1. 2. Effect of HPTE on TI cell progesterone and androgen production

Cells ($1 \times 10^5/\text{ml}$) 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 as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment. (a, c), $P < 0.01$ and $P < 0.001$, respectively, when compared with the appropriate control group.

Figure 1. 3. Effect of HPTE co-incubated with the substrate 22(OH)-cholesterol on TI cell progesterone and testosterone production

Cells (1×10^5 /ml) were exposed to varying concentrations of HPTE (0, 10-100 nM) and 22(OH)-cholesterol (10 μ M) at the time of plating for 24 h. HPTE and 22(OH)-cholesterol were dissolved in DMSO and ethanol, respectively. All treatment groups contained 0.1 % of each vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment. (b, c), $P < 0.01$ and $P < 0.001$, respectively, when compared with the appropriate control group.

Figure 1. 4. Effect of HPTE co-incubated with the substrate pregnenolone on TI cell progesterone and testosterone production

Cells (1×10^5 /ml) were exposed to varying concentrations of HPTE (10-100 nM) and pregnenolone (10 μ M) at the time of plating for 24 h. HPTE and pregnenolone were dissolved in DMSO and ethanol, respectively. All treatment groups contained 0.1 % of each vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment.

Figure 1. 5. Effect of HPTE co-incubated with the substrate progesterone on TI cell testosterone production

Cells ($1 \times 10^5/\text{ml}$) were exposed to varying concentrations of HPTE (10-100 nM) and progesterone (10 μM) at the time of plating for 24 h. HPTE and progesterone were dissolved in DMSO and ethanol, respectively. All treatment groups contained 0.1 % of each vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment

Figure 1. 6. Effect of HPTE co-incubated with the substrate androstenedione on TI cell testosterone production

Cells ($1 \times 10^5/\text{ml}$) were exposed to varying concentrations of HPTE (10-100 nM) and androstenedione ($10 \mu\text{M}$) at the time of plating for 24 h. HPTE and androstenedione were dissolved in DMSO and ethanol, respectively. All treatment groups contained 0.1 % of each vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment.

Figure 1. 7. Effect of HPTE co-incubated with the exogenous testosterone as substrate on 17 β -estradiol production by cultured GC

Cells (1×10^5 /ml) were exposed to varying concentrations of HPTE (10-100 nM) and testosterone (10 μ M) at the time of plating for 24 h. HPTE and testosterone were dissolved in DMSO and ethanol, respectively. All treatment groups contained 0.1 % of each vehicle as a final concentration. Each treatment group represents the mean $\pm$ S.E.M. of at least three separate samples from a single experiment.

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**STUDY 2: THE METHOXYCHLOR METABOLITE, HPTE,
DIRECTLY INHIBITS THE CATALYTIC ACTIVITY OF
CHOLESTEROL SIDE-CHAIN CLEAVAGE (P450SCC) IN
CULTURED RAT OVARIAN CELLS**

Abstract

Both methoxychlor and its proposed active metabolite HPTE have weak estrogenic and antiandrogenic activities, and these effects are thought to be mediated through the estrogen receptor and androgen receptor, respectively. In the first study, we showed indirectly that HPTE inhibits the P450 cholesterol side-chain cleavage (P450_{scc}) step resulting in decreased progesterone, androgen and estrogen production by cultured ovarian 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 inhibited progesterone formation and the P450_{scc} catalytic activity in a dose-dependent manner with significant declines starting at 50 nM. However, HPTE did not change mRNA levels of the P450_{scc} system (P450_{scc}, adrenodoxin reductase and adrenodoxin). Of interest, estradiol, xenoestrogens (bisphenol-A or 4-tert-octylphenol), a pure anti-estrogen (ICI 182,780), or anti-androgens (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 reveal that HPTE directly inhibits P450_{scc} catalytic activity resulting in decreased progesterone production by cultured ovarian 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.

1. Introduction

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) [1]. In female rodents, exposure to MC or HPTE caused a uterotrophic response and the proliferation of ovarian surface epithelium through estrogen receptor (ER)-linked mechanisms [2-4]. Both MC and HPTE have weak estrogenic and antiandrogenic activities [5-7]. However, HPTE binds with higher affinity to the ER [1] and is a more potent androgen receptor (AR) blocker [7]. In human hepatoma cells (HepG2), HPTE acted as an ER α agonist; however, it was antagonistic to ER β and AR [8]. Although it was 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 [9]. For example, Muro 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 [9].

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 [10-12], and the effects of HPTE on testis steroidogenesis localized to the P450 cholesterol side-chain cleavage (P450_{scc}) step in the pathway of androgen production from cholesterol [9,12-14]. In the first study, we showed that HPTE inhibited the P450_{scc} step resulting in decreased progesterone,

androgen and estrogen production by cultured ovarian cells. It has not 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., Scottsdale, 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 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. [2,3,6,7-³H(N)]-Testosterone (specific activity 100 Ci/mmol), 25-[26,27-³H]-hydroxycholesterol (specific activity 80 Ci/mmol), and ¹⁴C-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. 17 β -estradiol (estradiol) was from Steraloids, Wilton, NH, USA. 4, 4'-Isopropylidenediphenol (bisphenol A) and 4-tert-octylphenol (octylphenol) were from Aldrich Chem. Co., Milwaukee, WI. 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/0.1ml). Animals were treated between 0800 and 1000 h each day. Approximately 48 h following exposure to PMSG, animals were sacrificed by intra-peritoneal pentobarbital injection (~200 mg/kg).

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 [15,16]. Briefly, the ovaries 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 x 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 x g) for 10 min to yield TI cells. The final pellet was then resuspended in DMEM/F-12 medium.

GC and TI cells (10⁵/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 NaHCO₃, 100 U/ml penicillin G and 100 µg/ml streptomycin as described previously [15]. 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 % CO₂. 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 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

P450_{scc} activity of intact TI cells and GC was determined by measuring the conversion of 25-[26,27-³H]-hydroxycholesterol to pregnenolone and ³H-labeled side-chain by utilizing a previously described procedure [17] with slight modifications [13]. In brief, isolated TI cells (2 x 10⁵/0.5 ml medium) or GC (5 x 10⁵/0.5 ml medium) were plated into 24-well culture plates. Varying concentrations of HPTE (0, 10, 50, or 100 nM) were added at the time of plating, and after exposure for 8 h, 25-[26,27-³H]-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% CO₂. Reactions were stopped by adding 50 µl of 1N NaOH to each well. The contents of each well were transferred to 7 ml borosilicate glass vials, and each well was washed with 1.05 ml 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 ³H-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 ³H-labeled substrate and unlabeled steroid product(s) (e.g.,

pregnenolone). The aqueous phase and the neutral alumina were mixed, and the tubes were centrifuged ($\sim 1640 \times g$) 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 using the 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 μ g) was reverse transcribed, using Superscript II (Life Invitrogen, Gaithersburg, MD). The cDNA generated was diluted 1:100, and 7.25 μ l 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 in the present case), and deriving the fold increase compared to the control, unstimulated cells (Table 2.1).

2.8. Statistical Analysis

Data were expressed as the mean $\pm$ 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

Because the previous *in vitro* study on rat TI cells and GC demonstrated that the P450scc step in the pathway of cholesterol conversion to progesterone and estrogen production was inhibited by exposure to HPTE (study 1), the current studies evaluated whether *in vitro* exposure to HPTE would directly alter P450scc catalytic activity.

To confirm that HPTE inhibits progesterone production, TI cells or GC were exposed to HPTE (0, 10, 50, 100 or 500 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. 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 (Figure 2.1).

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 or 100 nM) and 25-[26,27- ^3H]-hydroxycholesterol (0.5 μCi , 5 μM) as described in the methods to quantitate the release of ^3H -labeled side-chain into the medium. Control TI 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 (Figure 2.2). There was no measurable decline in the P450scc activity of GC following exposure

to 10-500 nM HPTE. Possibly, this was due to the much lower P450_{scc} activity and reduced sensitivity of the assay in GC. There was about a 21-fold difference between P450_{scc} 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, Figure 2.2).

3.3 Effect of HPTE on P450_{scc} Gene Expression

P450_{scc} 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) [19] 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 P450_{scc} is localized [19]. To evaluate whether HPTE alters mRNA levels of the P450_{scc} 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 P450_{scc}, ADN, ADR or StAR. Glyceraldehyde 3-phosphate dehydrogenase (GAPD4) was used as the reference standard (Figure 2.3).

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 ER α agonist [8], 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 17 β -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 17 β -estradiol (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, Figure 2.4, 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 (Figure 2.4, 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 (Figure 2.4, 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, Figure 2.4, 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 (Figure 2.4, Panel C).

Over all, these studies show that 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 of the “pure” estrogen antagonist ICI 182,780 (ICI) which binds to both ER α and ER β [20].

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 (Figure 2.5). 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 μ M) for 24 h. The progesterone level in control TI cells was 24.13 ± 1.37 ng/ml/ 10^5 cells (Figure 2.6). 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 (Figure 2.6).

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. Progesterone levels in control TI and GC were 20.79 ± 3.11 and 19.81 ± 3.53 ng/ml/ 10^5 cells, respectively (Figure 2.7, Panel A).

Exposure to flutamide alone (10-1000 nM, Figure 2.7, 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 (Figure 2.7, 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.

Discussion

Data obtained in this study indicate that the active metabolite of MC, HPTE, directly inhibits the catalytic activity of P450scc resulting in decreased progesterone production by cultured TI cells and GC from PMSG-primed immature rats. 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 by HPTE suggests that this is a direct effect on the enzyme, which is also confirmed by unchanged mRNA levels of the three components of the P450scc system (P450scc, ADR and ADN) in TI cells following exposure to 100 nM HPTE. Furthermore, this effect of HPTE action does not appear to be mediated through the ER or AR signaling pathways, the currently recognized modes of action of MC and HPTE. 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 (17 β -estradiol, bisphenol-A or octylphenol), antiestrogen (ICI) or antiandrogen (flutamide or the vinclozolin metabolite, M-2) when added alone to TI cells or to GC, mimicked the action of HPTE on progesterone production. These results suggest that the effect of HPTE on TI cell or GC progesterone formation are not due to its estrogenic, antiestrogenic or antiandrogenic qualities.

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 μ M HPTE for two days [21]. In this study, lower concentrations of HPTE (0.1 or 1 μ M) were without effect [21]. In

addition, the mRNA levels of 3 β -hydroxysteroid dehydrogenase and P450 aromatase also were reduced in rat GC following treatment with 10 μ M HPTE [21]. 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 μ M (data not shown).

Although there is general agreement that HPTE inhibits the P450_{scc} step, the mechanism of action is not established. Because HPTE acts as an ER α agonist but is antagonistic to ER β and AR, the receptor-linked actions of HPTE are plausible [8]. However, in the current study none of the estrogenic agents including 17 β -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 [9], 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 inclusion of ICI blocked the effects of HPTE [14]. We are unable to explain these differences in the results with ICI at the present time.

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 likely that this was due to the lower activity of the enzyme and reduced sensitivity of the assay in GC. This effect of HPTE on P450scc activity in TI cells is in agreement with the results of previously reported 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 [9,12,13]. Although how HPTE specifically inhibits this step in the steroidogenic pathway 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: 20 α -hydroxylation, 22-hydroxylation and the scission of the C20-22 carbon bond, and these steps occur within the single substrate-binding pocket of P450scc [18]. To our knowledge, there is 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 [22]. 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 [23]. In this study, the inhibition by MC was diminished by inclusion of exogenous cholesterol. However, in study 1, the addition of cholesterol as substrate did not prevent the inhibition of HPTE on TI cell progesterone formation. Another possible mode of action of HPTE is

to alter the electron transport system. P450_{scc} must receive electrons from NADPH through the intermediacy of two electron transfer proteins, adrenodoxin reductase (ADR, a flavoprotein) and adrenodoxin (ADN, an iron/sulfur protein) [18]. It has been reported that the covalent binding of MC to liver microsomal proteins involves the generation of several reactive oxygen species [24], 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 [22]. However, Muroso 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 [9,13].

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 P450_{scc} activity, which was the result of the decline of P450_{scc} mRNA levels, suggesting that enzyme activity declined due to reduced protein synthesis [14]. However, in the current study mRNA levels of P450_{scc} in TI cells were not affected by HPTE, although progesterone levels and P450_{scc} activity declined. It was also reported that HPTE inhibited androgen formation by cultured Leydig cells as early as one hour following exposure to HPTE [13]. 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 P450_{scc} activity is suppressed in steroidogenic cells by HPTE because the half life of P450_{scc} is estimated to be greater than 4 h [25]. Although the decline in progesterone

formation in rat ovarian 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.

In conclusion, this study suggests that HPTE directly inhibits P450scc catalytic activity in rat ovarian cells resulting in decreased progesterone production. 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.

Tables

Table 2.1. Primer sets used for Quantitation of mRNAs by real-time RT-PCR

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

Number in parentheses represents gene accession number

Figures

Figure 2. 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 $\pm$ S.E.M. of four samples from at least three separate experiments. (b, c), $P < 0.01$ and $P < 0.001$, respectively, when compared with the appropriate control group.

Figure 2. 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- ^3H]-hydroxycholesterol (0.5 uCi, 5 uM) for the last 16 h. The release of ^3H -labeled side-chain (^3H -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 $\pm$ S.E.M. of four samples from at least three separate experiments. (c), $P < 0.001$ when compared with the TI cell control group.

Figure 2. 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 $\pm$ S.E.M. of four samples from three separate experiments.

Figure 2. 4. Effect of estrogen or estrogen agonists on progesterone formation by TI cells or GC

Cells ($1 \times 10^5/\text{ml}$) 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 $\pm$ S.E.M. of four samples from at least three separate experiments. (a, b), $P < 0.050$ and $P < 0.01$, respectively, when compared to the appropriate control group.

Figure 2. 5. 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 $\pm$ S.E.M. of four samples from at least three separate experiments.

Figure 2. 6. Effect of concomitant exposure to ICI and HPTE on progesterone formation by TI cells or GC

Cells ($1 \times 10^5/\text{ml}$) 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 $\pm$ S.E.M. of four samples from at least three separate experiments. (a, c), $P < 0.050$, and $P < 0.001$, respectively, when compared to the appropriate control group.

Figure 2. 7. 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 $\pm$ S.E.M. of four samples from at least three separate experiments.

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**STUDY 3: THE METHOXYCHLOR METABOLITE, HPTE,
INHIBITS RAT LUTEAL CELL PROGESTERONE
PRODUCTION**

Abstract

Our previous studies showed that HPTE specifically inhibited P450 cholesterol side-chain cleavage (P450_{scc}) activity resulting in decreased progesterone, androgen and estrogen production by cultured ovarian follicular cells. It is not known whether HPTE has any effect on progesterone formation by the corpus luteum. Following ovulation, follicular cells differentiate into non-dividing progesterone-producing luteal cells. The current studies examined the effect of HPTE on progesterone production by cultured luteal cells isolated following treatment of immature female rats with an ovulatory dose of pregnant mare serum gonadotropin. Luteal cells were isolated 2, 5 or 8 days following the estimated time of ovulation to examine whether the stage of the luteal phase affected its sensitivity to HPTE with respect to progesterone formation. Cultured luteal cells were exposed to HPTE (0, 10, 50, 100 or 500 nM) alone for 24 h. Luteal cells isolated 2 or 5 days post ovulation had reduced progesterone formation following exposure to 50 nM HPTE with progressive declines to < 12 % of control at 500 nM HPTE. Luteal cells isolated on day 8 post ovulation were sensitive only to the highest dose of HPTE. A decline in P450_{scc} activity in response to increasing concentrations of HPTE correlated with the decline in progesterone formation. These studies revealed that HPTE progressively inhibits rat luteal cell progesterone formation at concentrations as low as 50 nM, and that this decrease in progesterone was associated with a corresponding decline in P450_{scc} catalytic activity.

1. Introduction

Steroid hormone production by the ovary is essential for oocyte and follicle development as well as for the initiation and maintenance of pregnancy in mammals [1]. The ovary produces progestins, androgens and estrogens in a sequential manner, with the former serving as substrate for the subsequent steroid, and the levels secreted vary with the stage of follicle development [2]. The preovulatory follicle (multiple follicles in rodents) secretes mainly estrogens (estradiol and estrone) along with androgens (dehydroepiandrostenodione, androstenedione and testosterone), which reach higher levels shortly before ovulation, whereas the corpus luteum primarily produces progesterone along with estrogens [3]. Previous *in vivo* studies in female and male rodents reported decreased serum progesterone and androgen levels, respectively, following exposure to MC [4-6]. Studies 1 and 2 suggested that HPTE inhibited the catalytic activity of P450_{scc} resulting in decreased progesterone, androgen and estrogen formation in follicular cells (theca-interstitial and granulosa). 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 [7]. Unlike follicular cells, luteal cells are insensitive to cAMP [8] and predominantly express ER α suggesting that the regulatory mechanisms that control their function may not be the same as those that control follicle cell function [9]. 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., Scottsdale, 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 Alpha-dri (Shepherd 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-[26,27-³H]-Hydroxycholesterol (specific activity 80 Ci/mmol), and ¹⁴C-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.

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 the third day after PMSG treatment 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 [10].

2.4. Isolation and Incubation of Luteal Cells

Luteal cells were isolated by slight modifications of previously described methods for theca-interstitial cells [11]. Briefly, the ovaries were harvested 2, 5 or 8 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 the corpora lutea remained intact under these conditions. The ovarian tissue was washed twice with medium and incubated for 10 min (37⁰ 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 x 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 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 $\mu\text{g/ml}$ streptomycin as described previously [12]. 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 and luteal cell numbers per well were chosen based on preliminary studies conducted to determine the sensitivity of cultured luteal cells to the chemical.

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

P450_{scc} activity of intact luteal cells was determined by measuring the conversion of 25-[26,27- ^3H]-hydroxycholesterol to pregnenolone and ^3H -labeled side-chain by utilizing a previously described procedure [13] with slight modifications. In brief, following

isolation of luteal cells (2×10^5 /0.5 ml medium), cells 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- ^3H]-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 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 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 ^3H -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 ^3H -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 ($\sim 1640 \times g$) 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. Statistical Analysis

Data were expressed as the mean $\pm$ 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. HPTE Inhibits Progesterone Production

Studies were initiated to determine whether progesterone production by luteal cells was sensitive to HPTE and whether the day of isolation following ovulation affected its sensitivity to HPTE. Luteal cells were exposed to HPTE (0, 10, 50, 100 or 500 nM) alone for 24 h on post ovulation days 2, 5 or 8. HPTE progressively inhibited progesterone formation in luteal cells isolated 2 or 5 days following ovulation. Statistical declines were observed at 50 nM in both groups. In day-2 luteal cells, progesterone levels declined progressively from 206.05 ± 10.58 ng/ml/ 10^5 cells in the control group to 30.95 ± 5.33 ng/ml/ 10^5 cells (15 % of control) in cells treated with 500 nM HPTE (Figure, 3.1 Panel A). Similarly, in luteal cells isolated on day 5 following ovulation, the production of progesterone decreased from 205.40 ± 54.00 ng/ml/ 10^5 cells in the control group to 16.84 ± 2.10 ng/ml/ 10^5 cells (8 % of control) in cells treated with 500 nM HPTE (Figure 3.1, Panel B). In contrast, in luteal cells isolated on day 8 post ovulation, progesterone formation declined only following treatment with 500 nM HPTE. Progesterone levels declined from 253.09 ± 20.27 ng/ml/ 10^5 cells in control cells to 127.67 ± 19.03 ng/ml/ 10^5 cells (50 % of control, Figure 3.1, Panel C).

3.2 HPTE Inhibits the Catalytic Activity of P450scc

To demonstrate the direct effect of HPTE on P450scc activity, luteal cells isolated on day 5 following ovulation were exposed to HPTE (0, 10, 50, 100 or 500 nM) and 25-[26,27- ^3H]-hydroxycholesterol (0.5 μCi , 5 μM) as described in the methods section to quantitate the release of ^3H -labeled side-chain into the medium. Control cells released 6.21 ± 1.46

ng/16 h/10⁵ cells. A significant decline in luteal cell P450_{scc} activity was observed following exposure to 100 nM HPTE, which decline further to 2 % of control following exposure to 500 nM HPTE (Figure 3.2).

Discussion

Data obtained in this study indicate 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 P450_{scc} activity, the rate-limiting step in the biosynthesis of progesterone, androgens and estrogens. Inhibition of progesterone formation could be observed at 50 nM HPTE 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 inhibited only by 500 nM HPTE, suggesting that the corpus luteum becomes less sensitive to HPTE during this later phase. The expected life span of the rat corpus luteum following treatment of immature female rats with an ovulatory dose of PMSG is 10-12 days [10]. Currently, we do not have an explanation for this reduced sensitivity to HPTE during the later stage of luteal life; however, one possibility is the sustained high levels of progesterone secreted by the corpus luteum, which may be protective. For example, it was shown that intra-ovarian administration of progesterone antagonist mifepristone (RU486) inhibited luteal progesterone production in pregnant rats [14], and the administration of the synthetic progesterone, promegestone (R5020), increased progesterone formation in the cultured luteal cells from 19-day-pregnant rats [15].

The pattern of inhibition by HPTE on progesterone formation by luteal cells isolated during the early post ovulation phase (post ovulation days 2 or 5) is similar to the pattern observed previously in follicular cells (studies 1 and 2), suggesting that the luteinization

of follicular cells after ovulation does not alter its sensitivity to HPTE. The corpus luteum expresses high levels of key proteins involved in the uptake and transport of cholesterol which facilitates progesterone production [7]. In fact, the current study demonstrated that the capacity of luteal cells to produce progesterone ($> 200 \text{ ng/ml}/10^5 \text{ cells}$) is more than 10-fold higher than the capacity of theca-interstitial cells and about 40-fold higher than that of granulosa cells under similar culture conditions (study 1). Although luteal cells have this enhanced capacity to produce progesterone, exposure to 100 nM HPTE lowered progesterone output to $<20 \%$ of control cells in both follicular and luteal cells (isolated on day 5 post ovulation). This may suggest that competitive inhibition by HPTE to the cholesterol-binding site of P450_{scc} is an unlikely mechanism to inhibit the enzyme as suggested previously in bovine adrenocortical cells [16], because higher levels of HPTE would be expected to be required to inhibit the enzyme and progesterone formation in luteal cells. In support of this observation, in the first study we observed that the addition of exogenous 22-hydroxycholesterol to cultured theca-interstitial cells did not block the inhibition of progesterone formation elicited by HPTE.

The current study on rat luteal cells confirms the results of our previous studies that HPTE directly inhibits progesterone formation in both rat ovarian theca-interstitial and granulosa cells (studies 1 and 2). HPTE also has been reported to inhibit androgen formation in rat Leydig cells [17,18]. Similar to the studies reported in rat Leydig cells [18] and TI cells (study 1), the decline in androgen or progesterone formation, respectively, by HPTE was associated with a decline in P450_{scc} activity in luteal cells. Thus, HPTE appears to reduce steroid formation in different steroid-producing cells at a

common steroidogenic step. Previous studies in rat Leydig cells [19] and in rat ovarian GC [20] suggested that the decline in P450scc activity was due to the inhibition of new protein synthesis for the enzyme; however, in rat ovarian TI cells, HPTE had no effect on P450scc mRNA levels. We propose that similar to TI cells, MC / HPTE inhibits progesterone formation in rat luteal cells by directly inhibiting P450scc catalytic activity.

In conclusion, this study demonstrates for the first time that HPTE directly inhibits progesterone formation by rat ovarian luteal cells isolated from PMSG-treated immature rats. This decline in progesterone formation was associated with the inhibition in luteal cell P450scc activity.

Figures

Figure 3. 1. Effect of HPTE on progesterone formation by luteal cells isolated on post ovulation days 2, 5 or 8

Cells (1×10^5 /ml) were exposed to varying concentrations of HPTE (0, 10-500 nM) at the time of plating for 24 h. Luteal cells were isolated on post ovulation day 2 (Panel A), day 5 (Panel B) or day 8 (Panel C). HPTE was dissolved in DMSO, and all treatment groups contained 0.1 % of the vehicle. Each treatment group represents the mean $\pm$ S.E.M. of four samples from at least three separate experiments. (b, c), $P < 0.01$ and $P < 0.001$ respectively, when compared to the appropriate control group.

Figure 3. 2. Effect of HPTE on P450scc activity of rat luteal cells

Luteal cells ($2 \times 10^5/0.5$ ml), isolated on day 5 after ovulation, were exposed to varying concentrations of HPTE (0, 10-500 nM) for 24 h and 25-[26,27- ^3H]-hydroxycholesterol ($0.5 \mu\text{Ci}$, $5 \mu\text{M}$) for the last 16 h. The release of ^3H -labeled side-chain (^3H -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 $\pm$ S.E.M. of four samples from at least three separate experiments. (b, c), $P < 0.01$ and $P < 0.001$ respectively, when compared to the control group.

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**STUDY 4: THE EFFECTS OF *IN VIVO* EXPOSURE OF
METHOXYCHLOR TO IMMATURE RATS ON SERUM
PROGESTERONE AND ESTRADIOL LEVELS AND THE
EX VIVO FORMATION OF PROGESTERONE BY THECA-
INTERSTITIAL CELLS**

Abstract

In the first three *in vitro* studies, the exposure of isolated ovarian cells (theca, granulosa and luteal) with the proposed active metabolite of MC, HPTE, resulted in decreased progesterone and estradiol production, suggesting direct ovarian effects. The current study was initiated to examine whether the daily *in vivo* exposure of immature female rats to methoxychlor (0, 20-200 mg/kg) for a short duration (5-6 days), alone or under conditions where the maturation of ovarian follicles was accelerated by treating animals with pregnant mare serum gonadotropin (PMSG) during the last 2 days of exposure to methoxychlor, altered serum progesterone or estradiol levels or the *ex vivo* production of progesterone by theca-interstitial cells. In animals not treated with PMSG, serum progesterone levels declined progressively with the dose of methoxychlor starting at the 100 mg/kg dose; however, under these conditions, serum estradiol levels were below the level of detection. In those studies where animals were exposed to methoxychlor and to PMSG during the last 2 days of methoxychlor exposure, serum progesterone levels of methoxychlor-exposed animals were no different than control levels; however, serum estradiol level declined modestly in animals exposed to methoxychlor. In *ex vivo* studies on ovarian cells obtained from animals exposed *in vivo* to methoxychlor but without PMSG, progesterone production under basal conditions of cells exposed to methoxychlor was no different than control; however, when cells were exposed to hCG, cells exposed *in vivo* to methoxychlor actually produced more progesterone than control cells. In *ex vivo* studies on ovarian cells obtained from animals exposed *in vivo* to methoxychlor but also to PMSG during the last 2 days of methoxychlor exposure, ovarian cells incubated under basal conditions and exposed to methoxychlor produced more progesterone than

control cells. These studies demonstrate that the pattern of circulating progesterone or estradiol levels and ovarian cell production of steroids in response to *in vivo* exposure to methoxychlor is influenced by the maturational status of ovarian follicles and to possible differences in how female rats metabolize methoxychlor and /or major ovarian steroids. Further, these results suggest that the pattern of active steroids secreted *ex vivo* by ovarian cells in response to MC exposure may differ from their *in vitro* response to MC/HPTE.

1. Introduction

The ovary produces mainly two biologically active ovarian steroids, estradiol and progesterone along with less active or inactive steroid hormones such as pregnenolone and androstenedione [1]. Circulating levels of each of these hormones is regulated by the rate of ovarian secretion as well as peripheral metabolism by the liver, adipose tissue, skin, uterus and ovary [2-4]. The first three *in vitro* studies have suggested that MC directly targets ovarian steroidogenesis, whereby progesterone formation by ovarian cells was inhibited by the proposed active metabolite HPTE. In the very first study, HPTE inhibited progesterone, androgen and estradiol production by the whole follicle but the percent decrease in progesterone production was greater than the decline in estradiol and androgen levels. Previous *in vivo* studies in female and male rodents demonstrated decreased serum progesterone and androgen levels, respectively, following exposure to MC [5-7]. In male rats, the decline in serum androgen levels was correlated with a decrease in Leydig cell P450 cholesterol side-chain cleavage (P450_{scc}) activity, which converts cholesterol to pregnenolone in the pathway of androgen biosynthesis, and the *ex vivo* decline in testosterone formation by Leydig cells from the MC-exposed animals [7]. However, the decline in serum progesterone was not supported by *ex vivo* ovarian tissue steroid output from the same animals exposed to MC suggesting that peripheral metabolism of progesterone may influence serum steroid patterns [6]. In the same study, the administration of MC had no effect on serum estradiol levels; however, the *ex vivo* incubation of ovarian tissue isolated a day following treatment and incubated without or with hCG, demonstrated a reduction in estradiol production by cells exposed *in vivo* to MC [6]. In young mature female mice, the daily i.p. administration of MC for 20 days

had no effect on serum estradiol levels although atresia of antral follicles increased in relation to the dose of MC [8]. Thus, MC action on circulating ovarian steroid levels has not been clearly defined. The current studies evaluated whether the daily *in vivo* administration of methoxychlor (0, 20-200 mg/kg body weight) for a short duration (5 or 6 days) by gavage altered serum progesterone and estradiol levels and *ex vivo* theca-interstitial (TI) cell progesterone formation in immature female rats treated without or with pregnant-mare serum gonadotropin (PMSG) during the last 2 days of exposure to MC.

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., Scottsdale, 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 20 days when they were weaned. At this age, 2-3 pups were placed in a polycarbonate shoebox cage containing a mixture of Alpha-dri (Shepherd 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 corn oil were purchased from Sigma, St. Louis, MO, USA. Human chorionic gonadotropin (hCG, CR-127, 14900 IU/mg) was a gift from NIDDKD, Bethesda, MD. 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. Methoxychlor (MC) was from MP Biomedicals, Solon, OH, USA.

2.3. Treatment of Animals

Animals were 21-24 days of age at the start of treatment. They received 0, 20, 100 or 200 mg/kg body weight of MC dissolved in corn oil once daily by gavage. In those experiments where animals were treated with PMSG to stimulate the growth of follicles, they received a single injection (s.c.) of PMSG dissolved in PBS (20 IU/0.1ml) approximately 48 h prior to sacrifice. Under these conditions follicles (generally 7-9 per ovary in the current experiments) ovulate about 3 days after treatment with PMSG, and 2 days after treatment with PMSG when animals were sacrificed, follicles would consist of antral, preovulatory follicles. Fresh MC was prepared every other day during this treatment period. This dose range was selected because they have been used in previous studies and demonstrated to affect steroid production in female and male rats [6,7]. The volume of corn oil alone or MC in corn oil that each animal received was equivalent to 5 ml/kg body weight during this treatment period. Body weights were taken daily to adjust the volume or dose of corn oil or MC administered for any weight gains, and all treatment groups gained weight during this treatment period, and there was no statistically significant body weight change among dose groups. Animals were treated between 0800 and 1000 h each day. Approximately 24 h following the last MC exposure, animals were sacrificed by immersing each animal in a container saturated with CO₂. Trunk blood was collected. After sitting at room temperature for ~30 min, blood samples were centrifuged to obtain serum. Ovaries were collected for *ex vivo* studies. To reach the

adequate cell yield per group, ovaries from 2-3 animals as well as the blood samples from the same animals were pooled.

2.4. Isolation and Incubation of Theca-Interstitial Cells

Theca-interstitial (TI) cells were isolated by slight modifications of the methods described previously [9]. Briefly, the ovaries 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 granulosa cells. The residual ovarian tissue was washed twice with fresh medium and incubated in a rotary shaker bath for 10 min (37⁰ 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 granulosa cells, 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 x g) for 10 min to yield TI cells. The final pellet was then resuspended in DMEM/F-12 medium. TI cells (10⁵/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 NaHCO₃, 100 U/ml penicillin G and 100 µg/ml streptomycin as described previously [10]. Cells were cultured for 24 h in DMEM / F12 medium at 37⁰ C in a humidified atmosphere of 95 % air and 5 % CO₂.

2.5. Measurement of Progesterone and Estradiol

Progesterone and estradiol were quantitated directly from the collected medium by radioimmunoassay (RIA) as described in the manufacturer's protocol (Diagnostic Products Corp., Los Angeles, CA).

2.6. Statistical Analysis

Data were expressed as the mean $\pm$ 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. Serum Progesterone and Estradiol Levels

To evaluate whether MC affects serum progesterone or estradiol levels, 21-24 day old animals were exposed once daily to 0, 20, 100 or 200 mg/kg of MC for 6 consecutive days. Serum progesterone levels declined progressively from 3.32 ± 0.64 ng/ml in control animals to 1.42 ± 0.21 ng/ml in animals exposed to 100 mg/kg MC (~43 % of control) and decline further to 1.07 ± 0.26 ng/ml (~30 % of control) in animals treated with 200 mg/kg of MC (Figure 4.1). Serum estradiol levels were below sensitivity of the RIA under these conditions and could not be quantitated.

To examine whether MC affects serum progesterone and estradiol levels in PMSG-primed animals, 21-24 day-old animals were exposed once daily to 0 or 200 mg/kg MC for 6 consecutive days, and each animal received a single injection of PMSG (20 IU/0.1 ml, s.c.) ~ 48 h prior to the time blood collection to stimulate the growth of follicles. Serum progesterone levels were unaffected in the animals exposed to MC (4.97 ± 0.58 ng/ml in control animals, Figure 4.2, Panel A). Because ovarian follicle maturation was enhanced by treatment with PMSG, serum estradiol could be quantified and declined from 299.98 ± 22.25 pg/ml in control animals to 214.05 ± 20.49 pg/ml (~72 % of control) in the animals exposed to 200 mg/kg of MC (Figure 4.2, Panel B).

3.2. *Ex vivo* Theca-Interstitial Cell Progesterone Formation

Because previous *in vitro* studies demonstrated that progesterone formation by cultured theca-interstitial (TI) cells was inhibited by exposure to HPTE (studies 1 and 2), the current studies evaluated whether the *in vivo* exposure of immature female rats to the parent compound, MC, without or with PMSG (a single injection ~ 48 h prior to animal sacrifice to stimulate the growth of follicles), would inhibit *ex vivo* progesterone formation by TI cells.

TI cells isolated from control animals produced 1.82 ± 0.28 ng/ml/ 10^5 cells under basal conditions (Figure 4.3, Panel A). Exposing animals to 100 mg/kg of MC for 5 consecutive days had no effect on TI cell progesterone formation under basal conditions. However, when TI cells, isolated from animals treated with 100 mg/kg MC for 5 consecutive days without PMSG, were cultured in the presence of 10 mIU/ml hCG, control cells produced 12.53 ± 1.28 ng progesterone /ml/ 10^5 cells (~ 7 fold higher than cells cultured under basal conditions), and TI cells isolated from animals exposed to 100 mg/kg MC produced even more progesterone (26.44 ± 1.08 ng/ml/ 10^5 cells; 211 % of control; Figure 4.3, Panel B).

Culture of TI cells isolated from animals exposed *in vivo* to MC (0 or 200 mg/kg) for 6 consecutive days, with the addition of PMSG during the last 2 days of exposure, is presented in Figure 4.3, Panel C. These cells were not exposed to hCG because they were exposed to PMSG *in vivo*. TI cells from control animals produced 25.30 ± 2.09 ng/ml/ 10^5 cells of progesterone over 24 h. Progesterone levels increased to 38.44 ± 3.27 ng/ml/ 10^5 cells (152 % of control) in TI cells exposed *in vivo* to 200 mg/kg MC.

Discussion

The current studies have demonstrated that the *in vivo* exposure of MC (0, 20-200 mg/kg) to immature female rats for a short duration (5-6 consecutive days) progressively lowers serum progesterone levels. This decline could be observed at a dose of 100 mg/kg body weight, which declined further at the 200 mg/kg dose. In contrast, when immature female rats were exposed to MC (200 mg/kg) for 5 consecutive days and the maturation of ovarian follicles accelerated by treating animals with PMSG during the last 2 days of MC exposure, serum progesterone levels were unaltered, although serum estrogen levels declined to ~ 72 % of control. Furthermore, when TI cells, isolated from immature female rats previously exposed *in vivo* to corn oil or MC (100 mg/kg) alone for 5 consecutive days, were cultured for 24 h, progesterone levels were no different than control in TI cells exposed *in vivo* to MC and cultured without hCG. However, when these cells were cultured in the presence of 10 mIU/ml hCG, TI cells that were exposed *in vivo* to MC actually produced more progesterone (211 % of control). In addition, when TI cells, isolated from immature rats exposed to corn oil or 100 mg/kg MC for 6 consecutive days and to PMSG during the last 2 days of exposure, were cultured in the absence of hCG, TI cells exposed *in vivo* to MC produced more progesterone than control cells (152 % control).

In the first two *in vitro* studies, the exposure of isolated GC or TI cells from 2-day PMSG-primed immature female rats directly with the reported active metabolite HPTE (0, 10-100 nM) for 24 h resulted in the progressive decline in progesterone formation

(studies 1 and 2). These observations suggested that HPTE directly inhibits progesterone formation by rat ovarian cells, and this decline in progesterone corresponded to a decline in P450 cholesterol side-chain cleavage (P450_{scc}) activity. Based on these results the current studies were conducted to determine whether the *in vivo* exposure of immature female rats to MC, the parent compound to HPTE, for a short duration (5 or 6 days) would lower serum progesterone or estradiol levels and/or inhibit *ex vivo* ovarian cell progesterone formation. In immature animals not primed with PMSG, the *in vivo* exposure to MC (0, 20, 100 or 200 mg/kg) progressively reduced serum progesterone at the 100 and 200 mg/kg doses in the current studies. Under the conditions used in this study where follicular maturation was not enhanced by treating animals with PMSG, most of the progesterone would be expected to originate from theca cells of maturing non antral follicles. In a previous study where MC (0, 100 - 500 mg/kg) was administered by gavage to adult female rats on days 1-8 of pregnancy, serum progesterone levels also decline beginning at the 100 mg/kg dose [5]; however, under these conditions, most of the progesterone would have originated from luteal tissue. In a follow-up study, adult female rats were exposed daily to *in vivo* MC (0, 25-500 mg/kg) during the first 8 days of pregnancy and serum and the *ex vivo* production of progesterone and estradiol by ovarian cells (which would consist mainly of luteal cells) was determined [6]. Similar to the initial study, serum progesterone levels declined starting at the 50 mg/kg dose, then levels were progressively lower at higher doses of MC; however, serum estradiol levels were unaffected by exposure to MC. When minced ovarian tissue from these animals was incubated without or with hCG for 1 h, progesterone production in the tissue exposed *in vivo* to MC, was no different than control; however, both basal and hCG-stimulated

testosterone and estradiol formation from these same ovarian tissue declined starting at the exposure dose of 200 mg/kg MC [6]. This pattern of circulating and ovarian steroid levels suggested that the decline in serum progesterone was due to enhanced peripheral metabolism of progesterone by MC exposure and not from a direct effect on ovarian progesterone formation, while the pattern of ovarian testosterone and estradiol formation suggested that *in vivo* MC exposure may have direct effects on ovarian steroid production at a step(s) following progesterone formation. These observations may, in part, explain the pattern of steroid levels in serum and in incubated ovarian tissue following *in vivo* MC exposure of pregnant rats, but this is not adequate to explain the pattern of circulating steroid levels and ovarian cell steroid formation in the current studies.

The observations that serum progesterone levels progressively declined in response to *in vivo* exposure to MC in the present studies and our previous *in vitro* results on cultured ovarian cells, supported the idea that MC has direct effects on ovarian steroidogenesis. However, the *ex vivo* incubation of ovarian cells from these animals demonstrated no decline of progesterone production, and, when these cells were stimulated with hCG, ovarian cells exposed to MC *in vivo* actually produced more progesterone than control cells. These results suggested that the decline in circulating progesterone levels may have resulted, in part, from the peripheral metabolism of the steroid, especially in the liver. In this regard, one pathway for metabolism in rodents is by 6 β - or 16 α -hydroxylation of progesterone [3,4], which are mediated by CYP3A and CYP2B enzymes, respectively [11], and both of which are induced by MC [12]. Although the induction of hepatic CYP3A and CYP2B by MC may explain, in part, the enhanced peripheral metabolism of

progesterone, this does not explain why the *ex vivo* formation of progesterone by ovarian cells was unaffected by *in vivo* MC exposure. In adult male rats, *in vivo* exposure to MC resulted in a reduction in serum testosterone levels and a decline in the *ex vivo* Leydig cell testosterone formation and P450 cholesterol side-chain cleavage (P450_{scc}) activity [7], supporting *in vitro* studies where HPTE directly inhibited testosterone formation by cultured rat Leydig cells [13,14]. Although it is possible that less HPTE is available in female rats to affect ovarian steroid production, both male and female rat livers possess P450 enzymes capable of metabolizing MC to HPTE and to other metabolites, although the male liver was reported to be more active [15,16]. It is possible also that MC is processed differently in ovarian tissue so that less HPTE is available locally to affect progesterone formation.

Currently, we do not have an explanation for the *ex vivo* hCG-stimulated increase in progesterone formation by ovarian cells exposed *in vivo* to MC. In peripheral tissues such as the ovary and uterus, progesterone can be 'inactivated' by its conversion to 20 α -hydroxyprogesterone, which is a less active hormone, by 20 α -hydroxysteroid dehydrogenase [2]. Thus, in cultured FSH-primed rat granulosa cells, an increase in progesterone accumulation was associated with the inhibition of 20 α -hydroxysteroid dehydrogenase activity by prolactin [17]. The effect of MC on serum estradiol levels or ovarian cell estrogen formation have been mixed. In young mature female mice, the daily i.p. administration of MC for 20 days had no effect on serum estradiol levels even though atresia of antral follicles increased in relation to the dose of MC [8]. In pregnant rats, the administration of MC by gavage for 8 consecutive days during early pregnancy had no

effect on serum estradiol levels; however, the *ex vivo* incubation of ovarian tissue isolated a day following treatment and incubated without or with hCG, demonstrated a reduction in estradiol production by cells exposed *in vivo* to MC [6]. It is noteworthy that the animal models used in these studies to evaluate the effects of *in vivo* MC exposure on serum estradiol levels were different than the current studies. Because serum progesterone levels were unaffected in animals exposed to MC and to PMSG for the last 2 days of MC exposure, this would suggest that an enzymatic step following progesterone formation in the ovary was inhibited by MC to account for the decline in serum estradiol levels in the present studies. Another explanation to account for the decline in serum estradiol levels is that an enzyme(s) involved in the metabolism of estradiol was enhanced by MC exposure.

In summary, the results of the present study demonstrate that the *in vivo* exposure of immature female rats to MC (where follicle maturation was not accelerated by treatment with PMSG), progressively lowers circulating progesterone levels. However, in PMSG-primed immature rats exposed to MC, there is no change in serum progesterone levels, while serum estradiol levels declined modestly compared to control animals. In addition, the *ex vivo* formation of progesterone by TI cells from animals exposed to MC is higher than control cells in the presence of hCG. These results suggest that the maturational status of ovarian follicles may influence the overall effects of *in vivo* MC exposure on circulating steroid levels or of the steroid-producing capacity of ovarian cells. In addition, the effects of *in vivo* MC exposure on the peripheral metabolism of MC itself to potential active metabolites or of the metabolism of ovarian steroids to inactive products, may

affect changes in circulating steroid levels or the capacity of ovarian cells to secrete active steroids. These results suggest further that the pattern of active steroids secreted *ex vivo* by ovarian cells in response to MC exposure may differ from previously reported *in vitro* effects of MC.

Figures

Figure 4. 1. Effect of MC on serum progesterone levels in immature female rats

Animals (21-24 days of age) were exposed to MC (0, 20-200 mg/kg body weight) alone dissolved in corn oil by gavage once daily for 6 consecutive days. Each treatment group represents the mean $\pm$ S.E.M. of 5 samples (pooled blood from 2-3 animals per sample). (a), $P < 0.05$ when compared to the control group.

Figure 4. 2. Effect of MC on serum progesterone and estradiol levels in PMSG-primed rats

Animals (21-24 days of age) were exposed to MC (0 or 200 mg/kg body weight) dissolved in corn oil by gavage once daily for 6 consecutive days, and each animal received a single injection of PMSG (20 IU/0.1 ml, s.c.) ~ 48 h prior to blood collection. Each treatment group represents the mean $\pm$ S.E.M. of 5 samples (pooled blood from 2-3 animals per sample). (b), $P < 0.05$ when compared to the appropriate control group.

Figure 4. 3. Effect of *in vivo* MC exposure on *ex vivo* theca-interstitial cell progesterone production

Animals (21-24 days of age) were exposed by gavage to MC (0 or 100 mg/kg) for 5 consecutive days without PMSG (Panels A and B) or to MC (0 or 200 mg/kg) for 6 consecutive days plus a single s.c. injection of PMSG (20 IU/animal) given during the last 2 days of MC exposure (Panel C). Animals were sacrificed ~ 24 h following the last day of MC treatment and ovaries were removed for isolation of TI cells. TI cells were cultured for 24 h in the absence (Panel A and C) or presence of 10 mIU/ml hCG (Panel B). Progesterone released into the medium was quantitated by RIA. Each treatment group represents the mean $\pm$ S.E.M. of 4 samples from at least three separate experiments. (c) $P < 0.001$ when compared to the appropriate control group.

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GENERAL DISCUSSION

Results obtained from the first three *in vitro* studies demonstrated that HPTE directly inhibits progesterone formation by PMSG-primed rat ovarian cells, and this decline in progesterone corresponded to a decline in P450 cholesterol side-chain cleavage (P450_{scc}) activity, which converts cholesterol to pregnenolone in the pathway of progesterone and estrogen biosynthesis. In addition, the percentage decrease in progesterone production was greater than the declines in estradiol and androgen formation suggesting that HPTE may increase the estrogen/progesterone ratio. Further, this effect of HPTE action could be observed at environmentally relevant concentrations (10-50 nM) and does not appear to be mediated through the ER or AR signaling pathways, the currently recognized modes of action of MC and HPTE. The results of the last study suggested that the *in vivo* exposure to MC lowers circulating progesterone levels in the immature rats not treated with gonadotropins, finding that is consistent with HPTE actions in the *in vitro* studies. However, the involvement of gonadotropins leads to an *ex vivo* increase in progesterone production by isolated ovarian theca cells and no change in circulating progesterone levels. This suggests that the pattern of active steroids secreted *ex vivo* by ovarian cells in response to MC exposure differs from the *in vitro* effects of MC/HPTE.

The inhibition of HPTE does not appear to be cell specific since progesterone formation by GC, TI and luteal cells were all affected in the current studies. A recent study also reported that HPTE lowered FSH-stimulated progesterone formation by isolated GC from immature rats [55]. Besides follicular cells, previous *in vitro* studies on isolated rat Leydig cells demonstrated that basal and hCG-stimulated testosterone formation are

decreased by HPTE inhibition of P450scc activity. The parent compound of HPTE, MC, was also shown to inhibit P450scc activity in isolated mitochondria from the bovine adrenal cortex [90] and progesterone formation by porcine GC [56,57].

The present *in vitro* observations indicate that HPTE specifically inhibits the P450scc step in the pathway of converting cholesterol to androgens and estradiol without affecting other steps in the ovarian steroidogenic pathway. Our results support the observations of other studies reporting that HPTE did not affect StAR mRNA levels, the primary transporter of cholesterol into mitochondria in GC and Leydig cells [55,91]. 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 μ M HPTE for two days [55]. In this study, lower concentrations of HPTE (0.1 or 1 μ M) were without effect [55]. In addition, the mRNA levels of 3 β -hydroxysteroid dehydrogenase and P450 aromatase also were reduced in rat GC following treatment with 10 μ M HPTE [55]. In contrast, Zachow et al reported declines in estradiol formation and P450aromatase mRNA levels following exposure of rat GC to 10 μ M HPTE [55]. In the present *in vitro* studies, the conversion of testosterone to estradiol was not affected by 100 nM HPTE in GC suggesting that HPTE has no effect on aromatase activity. It should be noted that the concentration of HPTE used in the study by Zachow et al was three orders of magnitude higher than that used in the current studies.

Although there is general agreement that HPTE inhibits the P450_{scc} step, the mechanism of action is not established. Because HPTE acts as an ER α agonist but is antagonistic to ER β and AR, the receptor-linked actions of HPTE are plausible [54]. However, in the current study none of the estrogenic agents including 17 β -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 by HPTE of 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 [38], 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 inclusion of ICI blocked the effects of HPTE [91].

The current observations suggest that HPTE directly inhibits P450_{scc} catalytic activity, which, in turn, is responsible for a decline in progesterone formation by TI cells. This effect of HPTE on P450_{scc} activity in TI cells is in agreement with the results of previously reported 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 [37,38,58]. Although how HPTE specifically inhibits this step in the steroidogenic pathway is not known, a possible mode of HPTE action is to occupy the cholesterol-binding region of P450scc. To our knowledge, there is 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 [92]. 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 [90]. In this study, the inhibition by MC was diminished by inclusion of exogenous cholesterol. However, in our studies, the addition of cholesterol as substrate did not prevent the inhibition by HPTE of TI cell progesterone formation.

The observations that serum progesterone levels progressively declined in response to *in vivo* exposure to MC in the last study and the first three *in vitro* studies on cultured ovarian cells, supported the idea that MC/HPTE has direct effects on ovarian steroidogenesis. However, the *ex vivo* incubation of ovarian cells from MC-exposed animals demonstrated no decline in progesterone production, and, when these cells were stimulated with hCG, ovarian cells exposed to MC *in vivo* actually produced more progesterone than control cells. Under the conditions used in these studies where follicular maturation was not enhanced by treating animals with PMSG, most of the progesterone would be expected to originate from theca cells of maturing non-antral follicles. In a previous study where MC (0, 100 - 500 mg/kg) was administered by gavage to adult female rats on days 1-8 of pregnancy, serum progesterone levels also declined beginning at the 100 mg/kg dose [26]; however, under these conditions, most of the

progesterone would have originated from luteal tissue. In a follow-up study, adult female rats were exposed daily to *in vivo* MC (0, 25-500 mg/kg) during the first 8 days of pregnancy and serum and the *ex vivo* production of progesterone and estradiol by ovarian cells (which would consist mainly of luteal cells) was determined [29]. Similar to the initial study, serum progesterone levels declined starting at the 50 mg/kg dose, then levels were progressively lower at higher doses of MC; however, serum estradiol levels were unaffected by exposure to MC. When minced ovarian tissue from these animals was incubated without or with hCG for 1 h, progesterone production in the tissue exposed *in vivo* to MC, was no different than control; however, both basal and hCG-stimulated testosterone and estradiol formation from these same ovarian tissue declined starting at the exposure dose of 200 mg/kg MC [29]. Taken together, it is possible that the decline in circulating progesterone levels may have resulted, in part, from the peripheral metabolism of the steroid, especially in the liver. In this regard, one pathway for metabolism in rodents is by 6 β - or 16 α - hydroxylation of progesterone [86,87], which are mediated by CYP3A and CYP2B enzymes, respectively [88], and both of which are induced by MC [93]. Further, in peripheral tissues such as the ovary and uterus, progesterone can be 'inactivated' by its conversion to 20 α -hydroxyprogesterone, which is a less active hormone, by 20 α -hydroxysteroid dehydrogenase [85]. However, this does not explain why the *ex vivo* formation of progesterone by ovarian cells was unaffected by *in vivo* MC exposure. In adult male rats, *in vivo* exposure to MC resulted in a reduction in serum testosterone levels and a decline in the *ex vivo* Leydig cell testosterone formation and P450 cholesterol side-chain cleavage (P450_{scc}) activity [58], supporting *in vitro* studies where HPTE directly inhibited testosterone formation by cultured rat Leydig

cells [37,38]. Although it is possible that less HPTE is available in female rats to affect ovarian steroid production, both male and female rat livers possess P450 enzymes capable of metabolizing MC to HPTE and to other metabolites, although the male liver was reported to be more active [94,95]. It is possible also that MC is processed differently in ovarian tissue so that less HPTE is available locally to affect progesterone formation.

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