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In vivo exposure of young adult male rats to methoxychlor reduces serum testosterone levels and ex vivo Leydig cell testosterone formation and cholesterol side-chain cleavage activity

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Abstract

Methoxychlor (MC) was developed as a replacement for the banned pesticide DDT. After in vivo administration, it is metabolized in the liver to 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is proposed to be the active agent. Both MC and HPTE have been shown to exhibit weak estrogenic and antiandrogenic activities, and they are thought to exert their effects through estrogen and androgen receptors, respectively. Although in vitro studies using cultured rat Leydig cells have reported that HPTE inhibits both basal and hCG-stimulated testosterone formation, the response of circulating testosterone levels to in vivo MC has been more variable. Therefore, the current studies evaluated whether the daily in vivo administration of MC (0, 5, 40 and 200 mg/kg body weight) for a short duration (days 54–60 of age) by gavage altered serum testosterone levels and ex vivo Leydig cell testosterone formation in young adult male rats. These results demonstrate that both fluid-retained and fluid-expressed seminal vesicle weights declined to 44 and 60% of control, respectively, in the 200 mg/kg MC-exposed animals. Similarly, serum testosterone and dehydroepiandrosterone levels declined to 41 and 45% of control, respectively, in the 200 mg/kg MC-exposed animals; however, serum LH and FSH levels were unaffected. Ex vivo Leydig cell basal testosterone formation over 4 h declined to 49% of control in animals exposed to 200 mg/kg MC, and ex vivo Leydig cell P450 cholesterol side-chain cleavage activity declined to 79 and 50% of control in animals exposed to 40 and 200 mg/kg of MC, respectively, supporting previous in vitro studies which demonstrated the sensitivity of this step to MC.
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Keywords: Methoxychlor; Adult male rats; Testosterone

1. Introduction

Recent articles have proposed that the environmental or occupational exposure to endocrine-disrupting chemicals has altered the normal functioning of the endocrine system in humans and various wildlife species and that this has resulted in adverse reproductive effects [1,2]. Several reproductive trends that have occurred over the past 40–50 years as a result of these exposures in males include: (1) a world-side decline in semen quality [3], and (2) an increase in the prevalence of cryptorchidism [4], hypospadias [5] and testicular cancer [6]. However, the causal connection between exposure to potential endocrine-disrupting chemicals and the increased prevalence in male reproductive disorders is controversial and not fully accepted [7].

Methoxychlor (2,2-bis(*p*-methylphenyl)-1,1,1-trichloroethane; MC) is an insecticide which is currently in use on agricultural crops, especially following the ban of 2,2-bis(*p*-chlorophenyl)-1,1,l-trichloroethane (DDT) use in the United States in 1972. Its advantages over DDT are: (1) it is more rapidly metabolized and excreted in mammals [8], (2) it is acutely less toxic than DDT [9] and (3) it is more readily biodegraded [8]. The in vivo administration of MC to immature female rats increases uterine weight [10] and ornithine decarboxylase activity [11], which are both estrogen-associated responses. In addition, following in vivo administration of MC in rats, it is metabolized in the liver to 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is thought to be the active agent because it binds with

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higher affinity to the estrogen receptor (ER) than the parent compound [12]. However, other studies have demonstrated that MC, itself, has weak intrinsic estrogenic activity [13] and that it binds to both ER isoforms (ERα and ERβ) [14]. There are differences in the binding characteristics of HPTE to the two ER isoforms with HPTE acting as an agonist to ERα in human hepatoma cells (Hep G2), but acting mainly as a competitive antagonist to ERβ in this cell line [15]. In addition to exhibiting estrogenic activities, MC has been reported to act as an antiandrogen following in vivo exposure in male rats [16]. In Hep G2 cells transiently transfected with the androgen receptor (AR) and an androgen-responsive reporter, both MC and HPTE were antagonistic, although HPTE was approximately an order of magnitude more potent [17]. Thus, with respect to the specific type of reproductive effect that can be exerted by MC and HPTE, this can be influenced by the steroid receptor type (ER or AR) and the sub-type (ERα and ERβ) present in responsive cells. It should be noted that although blocking AR-mediated actions offers one mechanism to explain the antiandrogenic effects of MC and HPTE, other potential mechanisms include the inhibition of testosterone biosynthesis by Leydig cells, the inhibition of testosterone conversion to dihydrotestosterone (DHT) in DHT-sensitive tissues or the increased elimination of androgen.

Previous in vitro studies have reported that HPTE inhibits both basal and hCG-stimulated testosterone formation by isolated Leydig cells from immature [18] and adult [18,19] rats. However, the effects of in vivo MC exposure on circulating or interstitial fluid androgen levels or ex vivo testicular androgen production have been variable. Thus, the daily dosing of Long–Evans hooded male rats with 0, 25 or 50 mg/kg of MC by gavage from the day of weaning for 56 consecutive days had no effect on serum testosterone levels [20]. However, daily exposure of Long–Evans rats to higher doses of MC (0, 100 or 200 mg/kg) from the day of weaning until day 77–100 of age, reduced serum and interstitial fluid testosterone levels at the 100 mg/kg dose but not the 200 mg/kg dose and also decreased ex vivo hCG-stimulated testosterone formation of decapsulated whole testis at the highest dose [21]. It also has been reported that daily exposure by gavage of male Long–Evans hooded rats to 0, 200, 300 or 400 mg/kg of MC for a more extended period (from weaning to ∼11 months of age), did not significantly reduce serum or interstitial fluid testosterone levels, although seminal vesicle weights declined to 27% of control at the highest dose [16]. These differences in results among these studies may be related to differences in the dose and length of exposure to MC. Regarding the length of exposure, relatively long durations were used in these reports. To our knowledge, the effect of relatively short-term in vivo exposure to MC has not been studied in post-weaned male animals. The current studies evaluated the sensitivity of circulating testosterone levels and ex vivo Leydig cell testosterone production following exposure by gavage to MC at 0, 5, 40 or 200 mg/kg for seven consecutive days in young adult male rats. In addition, because the in vitro exposure of rat Leydig cells to HPTE was demonstrated previously to inhibit P450 cholesterol side-chain cleavage (P450scc) activity [18,19], the current studies evaluated whether in vivo exposure to MC altered ex vivo Leydig cell P450scc activity.

2. Materials and methods

2.1. Animals

Male rats (Sprague–Dawley certified virus free, Hla (SD)CVF) were purchased from Hilltop Lab Animals Inc., Scottdale, PA. They were purchased at 48 days of age and housed individually in polycarbonate shoebox cages 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), corn oil, methoxychlor (MC), 25-hydroxycholesterol, and neutral alumina were purchased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (BSA, clinical reagent grade) and ecolite (liquid scintillation fluid) were from ICN Pharmaceuticals Inc., Costa Mesa, CA. Diethyl ether and chloroform were from Fisher Scientific, Pittsburgh, PA. Dulbecco's Modified Eagle Medium (DMEM) without phenol red, Ham's F-12 nutrient mixture (F-12) without phenol red, medium 199 (Med 199), Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++}, sodium bicarbonate, soybean trypsin inhibitor, phosphate buffered saline (PBS) and *N*-2-hydroxyethylpiperazine-*N*′-2-2-ethane sulfonic acid (HEPES) were from Life Technologies, Grand Island, NY. [2,3,6,7-^{3}H(N)]-testosterone (specific activity ∼100 Ci/mmol), 25-[26,27-^{3}H]-hydroxycholesterol (specific activity ∼80 Ci/mmol), and ^{14}C-isocaproic acid were from Perkin-Elmer Life Sciences, Boston, MA. Testosterone was from Steraloids, Wilton, NH. Human chorionic gonadotropin (hCG, CR-127, 14900 IU/mg) was a gift from NIDDKD, Bethesda, MD. Radioimmunoassay (RIA) kits for rat FSH (RPA550) and LH (RPA552) were purchased from Amersham Biosciences, Piscataway, NJ. RIA kits to quantitate serum dehydroepiandrosterone (DHEA) and corticosterone were purchased from Diagnostic Products Corporation, Los Angeles, CA.

2.3. Treatment of animals

Animals were 54 days of age at the start of treatment. They received 0, 5, 40 or 200 mg/kg body weight of MC dissolved in corn oil once daily for seven consecutive days by gavage. Fresh MC was prepared every other day during this treatment period. This dose range was selected because these doses have been used in previous studies without demonstrating toxic effects in the exposed animals, and the 7-day exposure period was selected because longer exposure periods appeared to make the animals less sensitive in previous studies. The volume of corn oil alone or MC in corn oil that each animal received was equivalent to 5 ml/kg body weight. Body weights were taken daily to adjust the volume or dose of corn oil or MC administered for these weight gains, and all treatment groups gained weight during this treatment period. Animals were treated between 08:00 and 10:00 h each day. Approximately, 22 h following the last exposure, animals were sacrificed by immersing each animal in a container saturated with CO_2. Whole body weights were determined, and trunk blood was collected. After sitting at room temperature for ∼30 min, blood samples were centrifuged to obtain serum. Testicular (paired) and seminal vesicle (paired, both fluid-retained and fluid-expressed) weights were determined.

2.4. Isolation and incubation of Leydig cells

Animals were 61 days of age when sacrificed. Testes were decapsulated and digested in 0.25 mg/ml collagenase in Med 199 containing 0.1% BSA and 10 μg/ml DNase 1 for 20–30 min at 37 °C. The dissociated interstitial cells

Table 1
The effects of in vivo methoxychlor on whole body and organ weights

Final weights (g)	Methoxychlor (mg/kg)			
	0	5	40	200
Whole body	334.0 ± 3.8	343.9 ± 4.4	328.4 ± 4.4	314.8 ± 4.8 b
Testes	3.44 ± 0.06	3.36 ± 0.05	3.38 ± 0.04	3.25 ± 0.05
Seminal vesicles (fluid-retained)	0.97 ± 0.04	0.89 ± 0.04	0.82 ± 0.06	0.43 ± 0.04 c, f
Seminal vesicles (fluid-expressed)	0.52 ± 0.02	0.46 ± 0.02	0.47 ± 0.02	0.31 ± 0.02 c, f

These results are the mean ± S.E.M. of a minimum of 13 animals per treatment group. (b and c) $P < 0.01$ and $P < 0.001$, respectively, when compared to the appropriate control group. (f) $P < 0.001$ when compared to the immediate lower dose MC-treated group.

were layered over a 60% Percoll gradient and centrifuged at ∼25,000 × *g* for 1 h at 4 °C. Cells localizing between densities of 1.07 and 1.09 g/ml, which represented ∼60% Leydig cells, based on positive histochemical staining for 3β-hydroxysteroid dehydrogenase activity, were isolated.

Leydig 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, 100 μg/ml streptomycin and 0.1% BSA as described previously [19]. Cells were incubated at 33 °C for 4 h in a humidified atmosphere containing 5% CO_2 and 95% air, under basal conditions and following stimulation with 10 mIU/ml hCG. Following incubation, media were collected for quantitation of testosterone released into the medium.

2.5. Measurement of serum DHEA, corticosterone, FSH and LH

Serum DHEA levels were quantitated on diethyl ether-extracted samples by RIA following the vendor's instructions. Serum corticosterone, FSH and LH were quantitated directly from the serum as described in the manufacturers' protocol.

2.6. Measurement of testosterone

Testosterone released into the medium was quantitated by specific RIA as described previously [22]. Serum testosterone levels were quantitated by RIA following extraction with diethyl ether as described previously [22].

2.7. Measurement of P450 cholesterol side-chain cleavage activity

P450scc activity of intact Leydig cells was determined by measuring the conversion of 25-[26,27-^{3}H]-hydroxycholesterol to pregnenolone and ^{3}H-labeled side-chain by utilizing a previously described procedure [23] with slight modifications [19].

2.8. Statistical analysis

Data were expressed as the mean ± standard error of mean (S.E.M.) and analyzed by ANOVA. Differences among treatment means were determined using Student–Newman–Keuls' test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Changes in whole body and organ weights

Although there were no differences in starting body weights among all treatment groups (initial body weights were 305.5, 311.4, 305.8 and 310.7 g for the 0, 5, 40 and 200 mg/kg MC-treated groups, respectively) and animals within each treatment group gained weight during the 7-day treatment period, the final body weight of the 200 mg/kg treatment group was less than control (6%) (Table 1). Testicular weights among the four treatment groups were not significantly different; however, both fluid-retained and fluid-expressed seminal vesicle weights declined to 44 and 60% of control, respectively, in the 200 mg/kg MC-treated animals. Both fluid-retained and fluid-expressed weights were determined to assess whether any potential changes in seminal vesicle weights represented more than the amount of seminal fluid produced or retained by the glands.

3.2. Changes in serum hormone levels

Serum testosterone levels declined from 4.40 ± 0.52 ng/ml in control animals to 1.82 ± 0.27 ng/ml (∼41% of control) in the 200 mg/kg MC-treated group (Table 2). Similarly, serum DHEA levels declined from 0.11 ± 0.01 ng/ml in control animals to 0.05 ± 0.01 ng/ml (∼45% of control) in the 200 mg/kg MC-treated group. In addition, in the 40 mg/kg MC-treated group there was a tendency for both DHEA and testosterone levels to be lower than control. However, serum corticosterone levels in all MC-treated groups were no different than control. Neither LH nor FSH levels in the MC-treated animals were different than the control group.

Table 2
The effects of in vivo methoxychlor on serum hormone levels

Serum hormone levels (ng/ml)	Methoxychlor (mg/kg)			
	0	5	40	200
Testosterone	4.40 ± 0.52	4.94 ± 0.91	3.85 ± 0.77	1.82 ± 0.27 a, d
Dehydroepiandrosterone	0.11 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.05 ± 0.01 b
Corticosterone	50.61 ± 11.33	46.35 ± 8.50	53.87 ± 7.96	84.48 ± 19.40
LH	4.87 ± 0.16	4.87 ± 0.12	4.97 ± 0.24	4.44 ± 0.33
FSH	20.18 ± 0.89	20.60 ± 0.69	20.32 ± 0.94	20.41 ± 0.92

These results are the mean ± S.E.M. of a minimum of 13 animals per treatment group. (a and b) $P < 0.05$ and $P < 0.01$, respectively, when compared to the appropriate control group. (d) $P < 0.05$ when compared to the immediate lower dose MC-treated group.

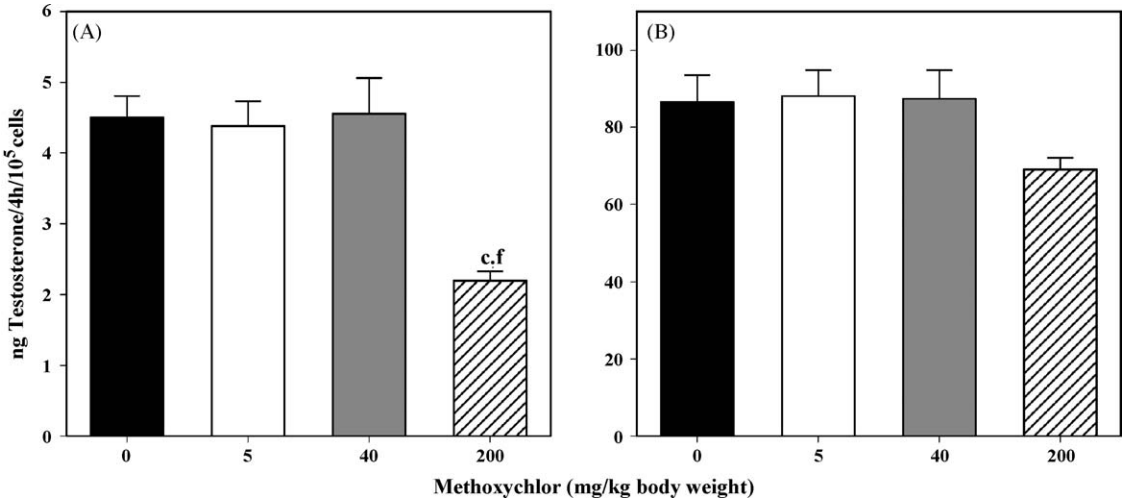


Fig. 1. The effects of in vivo methoxychlor on ex vivo Leydig cell testosterone production. These results are the mean ± S.E.M. of a minimum of 13 animals per treatment group. Panel A, testosterone formation under basal conditions; panel B, testosterone formation following treatment with 10 mIU/ml hCG. (c) $P < 0.001$ when compared to the appropriate control group. (f) $P < 0.001$ when compared to the immediate lower dose MC-treated group.

3.3. Ex vivo Leydig cell basal and hCG-stimulated testosterone levels

Because previous in vitro studies demonstrated that testosterone formation by cultured rat Leydig cells was inhibited by exposure to HPTE [18,19], the current studies evaluated whether the in vivo exposure of the parent compound, MC, to young adult male rats by gavage would inhibit the capacity of Leydig cells isolated from these animals (ex vivo studies) to synthesize testosterone. Leydig cells isolated from control animals produced 4.50 ± 0.31 ng testosterone/4 h/10^5cells under basal conditions (Fig. 1, panel A). Exposing animals to 5 or 40 mg/kg of MC for seven consecutive days had no effect on the ability of Leydig cells to synthesize testosterone; however, the exposure of animals to 200 mg/kg MC lowered the capacity of isolated Leydig cells to produce testosterone to 2.20 ± 0.13 ng/4 h/10^5 cells (∼49% of control).

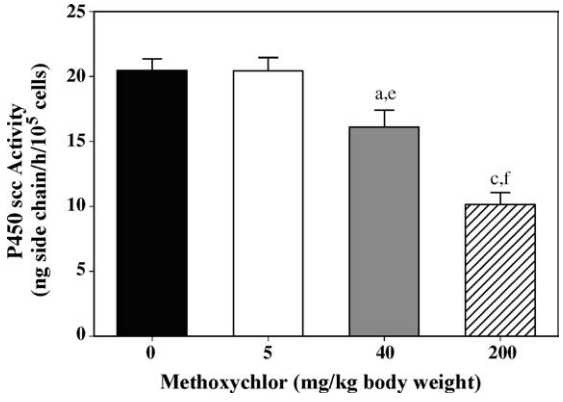


Fig. 2. The effects of in vivo methoxychlor on ex vivo Leydig cell P450scc activity. These results are the mean ± S.E.M. of a minimum of 11 animals per treatment group. The cell concentration was 10^5/0.5 ml DMEM/F-12 medium. The incubation time was 1 h at 33 °C. (a and c) $P < 0.05$ and $P < 0.001$, respectively, when compared to the control group. (e and f) $P < 0.01$ and $P < 0.001$, respectively, when compared to the immediate lower dose MC-treated group.

Leydig cells isolated from control animals and incubated in the presence of 10 mIU/ml hCG produced 86.52 ± 7.10 ng/4 h/10^5 cells (Fig. 1, panel B). This value is over 19-fold higher than the testosterone value of control cells incubated without hCG. Leydig cells isolated from MC-treated animals and incubated in the presence of hCG produced testosterone levels comparable to control, although the testosterone value of cells isolated from animals exposed to the 200 mg/kg dose of MC was ∼16% less than control.

3.4. Effect of MC on ex vivo Leydig cell P450scc activity

P450scc activity of Leydig cells isolated from control animals was 20.44 ± 0.92 ng side chain/h/10^5 cells (Fig. 2). P450scc activity of Leydig cells isolated from animals exposed to 5 mg/kg MC was no different than control; however, the enzyme activity of Leydig cells isolated from animals exposed to 40 mg/kg MC had reduced to 16.10 ± 1.29 ng side chain/h/10^5 cells (∼79% of control). P450scc activity of Leydig cells isolated from animals exposed to 200 mg/kg MC declined further to 10.15 ± 0.98 ng side chain/h/10^5 cells (∼50% of control).

4. Discussion

The current studies have demonstrated that the in vivo exposure of young adult male rats for seven consecutive days between days 54–60 of age with MC (5, 40 or 200 mg/kg body weight) inhibits Leydig cell testosterone biosynthesis at the highest exposure dose. This is reflected by a 59% decline in serum testosterone levels, a 56 and 40% decline in wet and expressed seminal vesicle weights, respectively, a 51% decline in ex vivo Leydig cell basal testosterone formation and 50% decline in ex vivo Leydig cell P450scc activity at the highest dose of MC.

These in vivo studies support previous in vitro studies on isolated Leydig cells from adult rats [18,19]. In these previous studies, cultured Leydig cells from normal rats were exposed to the reported active metabolite of MC, HPTE. The studies

by Akingbemi et al. suggested that the decline in testosterone formation following exposure to HPTE for 12 h was associated with a decline in P450scc mRNA levels [18]. However, in a later study, testosterone levels declined as early as 1 h following exposure of Leydig cells to HPTE, suggesting that HPTE may be acting directly on the catalytic activity of P450scc [19] because the half life of P450scc mRNA was reported to be ∼4–8 h [24], and, consequently, P450 protein and testosterone levels would not be expected to decline so rapidly. Although the current studies did not determine whether the decline in Leydig cell P450scc activity was due to a decrease in P450scc mRNA and the resultant reduction in P450scc protein levels, the fact that serum LH levels were unchanged in the current studies would suggest that the decline in Leydig cell testosterone biosynthetic capacity is due to the direct effects of MC and/or its active metabolite(s) on P450scc activity.

A previous in vivo study of daily MC administration (0, 25 or 50 mg/kg body weight) from weaning for 56 consecutive days reported no changes in serum testosterone, LH or FSH levels [20]. In a subsequent study, a wider range of MC doses (0, 25, 50, 100 or 200 mg/kg) was administered daily from weaning for nearly 3 months [21]. In that study, serum testosterone levels declined at the 100 mg/kg dose but not the 200 mg/kg dose, and interstitial fluid testosterone levels followed a similar pattern. HCG-stimulated testosterone formation of decapsulated whole testes from these animals was generally lower in animals exposed to the higher doses of MC, but there was variability in these results [21]. The fact that serum LH and FSH levels were unaltered in these animals suggested that MC was acting directly on the testis. Because of the variability in serum and testicular testosterone levels in response to 25–200 mg/kg MC, a follow-up study evaluated higher doses of MC (0, 200, 300, 400 mg/kg), administered daily from weaning for ∼10 months [16]. However, in that study there were no changes in serum LH and testosterone levels with any of the doses of MC. An explanation for the lack of change in serum androgen levels in these animals is not apparent; however, one explanation is that male rats become less sensitive to MC as they get older. In a later study by Okazaki et al., the daily gavage of 7-week-old male rats with 0, 20, 100 or 500 mg/kg MC for 28 days produced no change in serum testosterone levels at the two lower MC doses; however, levels declined at the 500 mg/kg dose [25]. It is of interest that seminal vesicle weights declined progressively with the dose of MC administered [16]. Because both MC and HPTE also exhibit antiandrogenic activity [17], the decline in seminal vesicle weights may represent antagonism to endogenous androgen(s) rather than to a decrease in circulating androgen. Whether the longer treatment times to MC could explain the lack of sensitivity of Leydig cells in the previous studies [16,21] is not known. Because MC is known to induce liver cytochrome P4502B and P4503A enzymes in the rat [26], it could affect its own metabolism as well as that of many endogenous substrates including testosterone [26,27]. However, how longer periods of exposure to MC would have less effect on Leydig cell testosterone formation or circulating androgen levels is not apparent at the present time.

Previous in vitro studies have suggested that P450scc activity of rat Leydig cells is sensitive to HPTE [18,19]. Consequently, the current studies evaluated whether serum levels of the main glucocorticoid, corticosterone, in the rat might be affected because P450scc activity of the adrenal cortex is the rate-limiting step in corticosterone biosynthesis in the rat. The present studies did not detect declines in serum corticosterone levels in response to in vivo MC. These results may suggest organ specificity in sensitivity of P450scc activity to MC or HPTE. Alternatively, the high variability in serum corticosterone levels (there was a >20-fold difference between the lowest and highest control values) may have limited our ability to detect any differences. Our ability to detect declines in serum DHEA levels in the current studies probably reflects the contribution of Leydig cells to circulating DHEA in the rat [28,29] and supports the sensitivity of Leydig cell P450scc activity to MC or HPTE. It is possible also that MC could damage P450scc catalytic activity by increasing the level of lipid peroxidation due to increased generation of oxygen free radicals [30]; however, a previous in vitro study reported that the concomitant inclusion of the antioxidants Trolox (a water-soluble derivative of vitamin E) or ascorbate with the MC metabolite, HPTE, did not protect cultured rat Leydig cells against the inhibitive effects of HPTE on testosterone formation [19].

Although the short-term exposure of male rats to MC in the current study reduced serum testosterone levels to ∼41% of control in animals exposed to 200 mg/kg MC, this did not produce the expected increase in serum LH levels. This may suggest that exposure to MC reduces hypothalamic and/or anterior pituitary sensitivity to the feedback effects of testosterone on LH secretion. It should be noted that in previous in vivo studies in which male rats were exposed to varying doses of MC for ∼2–10 months, no changes in serum LH and/or FSH were detected [16,20,21].

In summary, the results of the present studies demonstrate that the exposure of young adult rats to a short duration with MC lowers circulating testosterone levels and ex vivo basal Leydig cell testosterone formation in animals exposed to the 200 mg/kg dose. In addition, this decline in testosterone appears to be related to a decline in Leydig cell P450scc activity, which is reduced at the 40 mg/kg dose and progressively at the 200 mg/kg dose.

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