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ORIGINAL ARTICLE

Effects of methoxychlor and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane on 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase-3 activities in human and rat testes

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Summary

Human and rat testis microsomes were used to investigate direct inhibitory activities of methoxychlor (MXC) and its metabolite 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) on 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3). The 3β-HSD and 17β-HSD3 enzymes are involved in the reactions that culminate in androgen biosynthesis in Leydig cells. The results demonstrated that MXC and HPTE inhibited human 3β-HSD activity at a concentration of 10 nM. The half maximal inhibitory concentration (IC_{50}) for MXC inhibition of 3β-HSD was 53.21 ± 15.52 μM (human) and 46.15 ± 17.94 μM (rat), and for HPTE, it was 8.29 ± 2.49 μM (human) and 13.82 ± 2.26 μM (rat). At the higher concentration of 100 μM, MXC did not affect human and rat 17β-HSD3 activity. However, the IC_{50} for HPTE inhibition of 17β-HSD3 was 12.1 ± 1.9 μM (human) and 32 .0 ± 8.6 μM (rat). The mode of action of MXC and HPTE on 3β-HSD activity was non-competitive with the substrate pregnenolone, but was competitive with the cofactor NAD^+. The mode of HPTE inhibition of 17β-HSD3 was non-competitive with the substrate androstenedione, but was competitive with the cofactor NADPH. In summary, our results showed that HPTE, which is the biologically active metabolite of MXC, has the capacity for direct inhibition of 3β-HSD and 17β-HSD3 enzyme activity. Inhibition of enzyme activity is presumably associated with suppression of steroidogenesis in gonadal tissues and has implications for testis function.

Introduction

Methoxychlor (MXC), an organochlorine pesticide, is widely used to preserve fruits, crops, vegetables and home gardens. MXC is structurally similar to DDT, which has been characterized as a persistent organic pollutant. MXC is considered the preferred alternative to DDT for reasons of its low toxicity and lower levels of persistence in the environment (with a half-life of about 6 months). Therefore, it has gradually replaced DDT for use in insect control programmes and to preserve fruits and vegetables (Gupta *et al.*, 2006).

Previous observations indicate that MXC is a reproductive toxicant associated with oestrogenic activity (Hall *et al.*, 1997). After ingestion, MXC is metabolized into predominantly mono- and bis-demethylated metabolites, including 2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)-1,1,1-trichloroethane (OH-MXC) and 2,2-*bis*(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE; Kapoor *et al.*, 1970). Interestingly, HPTE was found to exert a greater reproductive toxicity in both sexes than the parent compound MXC (Gupta *et al.*, 2007). In the female, HPTE inhibited follicle-stimulating hormone and dibutyl cAMP-stimulated steroidogenesis, by regulation of messenger RNA levels of steroidogenic enzymes in rat granulosa cells (Harvey *et al.*, 2009). In addition, HPTE directly stimulated anti-Mullerian hormone secretion in the ovary (Uzumcu *et al.*, 2006). In the male, we previously

demonstrated that HPTE inhibits the P450 cholesterol side-chain cleavage (P450scc) gene (*Cyp11a1*) expression in rat Leydig cells and decreased androgen production (Akingbemi *et al.*, 2000). There is evidence that MXC is metabolically converted to HPTE, which exerts its oestrogenic effects because MXC is known to have a weaker affinity (IC_{50} of 80–190 μM) than HPTE (IC_{50} = 0.8 μM) to bind to human oestrogen receptor-α Bolger *et al.*, 1998). However, it is not presently clear whether both MXC and its metabolite HPTE have the capacity to cause direct inhibition of steroidogenic enzyme activity.

Four enzymes are involved in the androgen biosynthetic pathway: P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase/17–20 lyase and 17β-hydroxysteroid dehydrogenase (17β-HSD3). The enzyme 3β-HSD catalyses formation of progesterone (P4) from pregnenolone (PREG), whereas 17β-HSD3 catalyses formation of testosterone (T) from androstenedione (DIONE). Thus, the two enzymes (3β-HSD and 17β-HSD) play important roles in Leydig cell testosterone production (Penning, 1997). In this study, we compared the potencies of MXC and HPTE in the inhibition of 3β-HSD and 17β-HSD3 enzyme activity in the human and rat testes and identified their modes of action.

Materials and methods

Materials

[1,2-^{3}H]DIONE and [^{3}H]PREG (specific activity, 40 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA, USA). Unlabelled DIONE, PREG, P4 and testosterone were obtained from Steraloids (Newport, RI, USA). MXC was purchased from Sigma (St Louis, MO, USA) and HPTE was a gift from Dr W. R. Kelce (Monsanto Company, St Louis, MO, USA). MXC and HPTE were prepared using ethanol as solvent. Male Sprague–Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee of Rockefeller University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Human male testes were obtained from National Disease Research Interchange (Philadelphia, PA, USA).

Preparation of microsomal protein for 3β-HSD and 17β-HSD3 assays

Microsomal preparations of human and rat testes as well as 3β-HSD and 17β-HSD3 assays were performed as described previously (Hu *et al.*, 2009). Briefly, testes from 250–300 g Sprague–Dawley male rats were homogenized in cold 0.01 mM PBS buffer containing 0.25 mM of sucrose and centrifuged at 700 ***g*** for 30 min. The supernatants were transferred to new tubes and centrifuged at 10 000 ***g*** for another 30 min. Supernatants from the latter procedure were centrifuged at 105 000 ***g*** for 1 h twice. Pellets were resuspended and protein concentrations were measured using the Bio-Rad Protein Assay Kit (cat# 500–0006; Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Microsomes were used for measurement of 3β-HSD and 17β-HSD3 enzyme activity.

3β-HSD or 17β-HSD3 activity assay tubes contained 0.2 μM of PREG plus 40 000 cpm [^{3}H]PREG or 0.1 μM of DIONE plus 40 000 cpm [^{3}H]DIONE, respectively. The substrate concentration was selected based on interstitial fluid androgen levels in the rat testis (Sriraman *et al.*, 2001). To determine half maximal inhibitory concentration (IC_{50}), the reactions were initiated by addition of human (50 μg) or rat (10 μg) testis microsomal protein to 0.2 mM of NAD^+ for 3β-HSD, and 90 μg of human or rat testis microsomal protein with 0.2 mM of NADPH for 17β-HSD3 in the presence of varying concentrations of MXC or HPTE. In all cases, the reactions were stopped by adding 2 mL of ice cold ether. The steroids were extracted, and the organic layer was dried under nitrogen. Steroids were separated chromatographically on thin layer plates in chloroform and methanol (97 : 3) and radioactivity was measured using a scanning radiometer (System AR2000; Bioscan Inc., Washington, DC, USA). The percentage conversion of substrate to product was calculated by dividing the radioactive counts identified as product by the total counts associated with substrate plus product.

Statistics

Assays were repeated four times. The IC_{50} was calculated using GraphPad (version 4; GraphPad Software Inc., San Diego, CA, USA) and non-linear regression of curve fit with one-site competition. Lineweaver–Burk analysis was used to determine the mode of inhibition. Data were subjected to analysis by one-way ANOVA followed by DUNCAN multiple comparisons to identify significant differences between groups when three and more groups were analysed. All data are expressed as means ± SEM. Differences were regarded as significant at $p < 0.05$.

Results

Effects of MXC on 3β-HSD and 17β-HSD3 activity

The enzyme activity of 3β-HSD, which catalyses conversion of PREG to P4, was measured in human and rat testis microsomes. Both MXC and HPTE inhibited 3β-HSD activities in human (Fig. 1A) and rat testes (Fig. 1B). The minimum effective concentrations of MXC

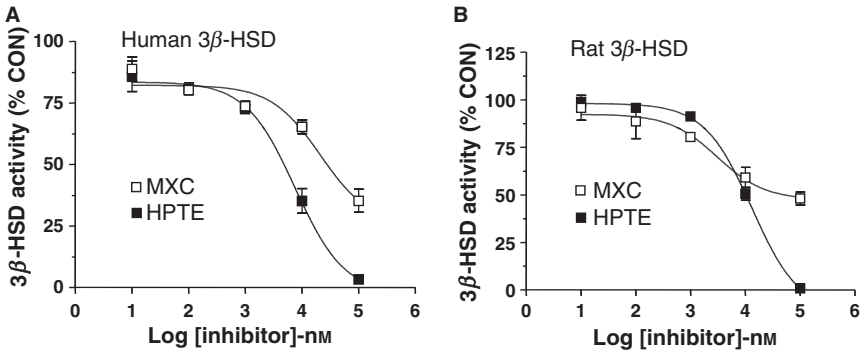

Figure 1 Inhibition of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity by methoxychlor (MXC) and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in human (A) and rat (B) testis microsomes. 3β-HSD activity was measured by the conversion of pregnenolone (PREG, 0.2 μM) to progesterone (P4). Conversion rate of control was 88.2 and 45.64% for the human and rat samples, respectively. IC_{50} values were calculated from these assays and are shown in Table 1 (mean ± SEM). Data represent mean ± SEM of four separate and independent experiments.

and HPTE inhibiting 3β-HSD activity were 10 nM (human) and 1 μM (rat; $p < 0.05$ compared with control). As shown in Table 1, the IC_{50} for inhibition of human 3β-HSD was 53.21 μM for MXC and 8.29 μM for HPTE, respectively. The IC_{50} for inhibition of rat 3β-HSD was 46.15 μM for MXC and 13.82 μM for HPTE, respectively.

At 100 μM, MXC did not inhibit human and rat 17β-HSD3 activity (Fig. 2), whereas HPTE significantly inhibited enzyme activity in both species. The IC_{50} of HPTE inhibiting human and rat 17β-HSD3 were 12.12 and 32.0 μM, respectively (Fig. 3 & Table 1). These data support observations indicating that MXC is metabolically activated to HPTE for inhibition of 17β-HSD3.

Modes of inhibition of MXC and HPTE on 3β-HSD and 17β-HSD3 enzyme activity

The mode of inhibition of 3β-HSD activity by MXC and HPTE was examined by analysis of enzyme kinetics. The results of Lineweaver–Burk plot analysis showed that both MXC and HPTE were non-competitive inhibitors of 3β-HSD in both human and rat testis microsomes (Fig. 4). However, when different cofactor (NAD^+) concentrations were used for measurement of 3β-HSD in rat testis microsomes (Fig. 5A,B), Lineweaver–Burk analysis showed that MXC and HPTE were competitive inhibitors of the cofactor NAD^+ (Fig. 5). Results indicated that MXC and HPTE competitively inhibited the 3β-HSD enzyme by competing with NAD^+ cofactor in its enzyme-binding site. These findings imply that MXC and HPTE exhibited similar modes of competitive inhibition for human 3β-HSD activity.

The mode of action of HPTE on 17β-HSD3 activity was also investigated by assessment of enzyme kinetics. The results of Lineweaver–Burk plot analysis showed that HPTE is a non-competitive inhibitor of 17β-HSD3 in both human and rat testis microsomes (Fig. 6A, human data; rat data not shown). Using different cofactor (NADPH) concentrations for assays of 17β-HSD3 activity in human and rat testis microsomal fractions (Fig. 6B), the Lineweaver–Burk analysis demonstrated that HPTE was a competitive inhibitor of the cofactor NADPH (Fig. 6B, human data; rat data not shown). These observations indicated that HPTE competitively inhibited 17β-HSD3 activity by competing with the NADPH cofactor in its enzyme-binding site.

Table 1 IC_{50} of inhibition of human and rat 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD3 by methoxychlor (MXC) and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)

	MXC		HPTE	
IC_{50} (μM)	Human	Rat	Human	Rat
3β-HSD	53.21 ± 15.52	46.15 ± 17.94	8.29 ± 2.49	13.82 ± 2.26
17β-HSD3	NI	NI	12.12 ± 1.94	32.00 ± 8.56

NI indicates no inhibition at 100 μM.

Discussion

The 3β-HSD and 17β-HSD3 enzymes are involved in critical reactions required for testosterone biosynthesis in testicular Leydig cells. Both enzymes are localized to intracellular microsomes and belong to the family of alcohol short-chain dehydrogenases (Penning, 1997). In this study, we observed dose-dependent inhibition of human and rat testis 3β-HSD by MXC and its metabolite HPTE. HPTE was about sevenfold more potent in the inhibition of human 3β-HSD activity than MXC, and was threefold more potent in the inhibition of rat 3β-HSD activity than MXC. Increased potency for HPTE has been attributed to

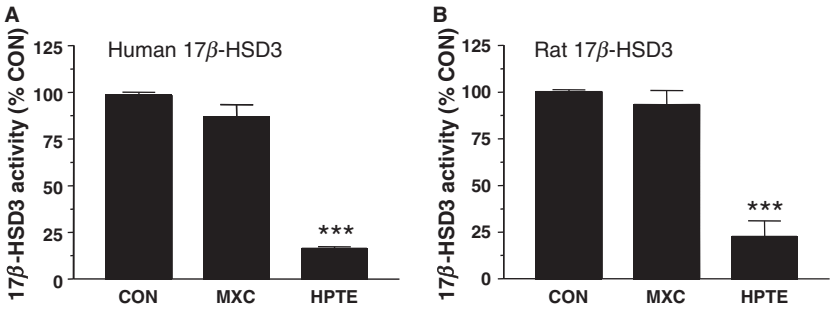

Figure 2 Inhibition of 17β-hydroxysteroid dehydrogenase (17β-HSD3) activity by methoxychlor (MXC) and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in human (A) and rat (B) testis microsomes. 17β-HSD3 activity was determined by conversion of androstenedione (100 nM) to testosterone in the presence or absence of 100 μM of MXC and HPTE (mean ± SEM). Conversion rate of control was 68.89 and 43.23% for the human and rat samples, respectively. Data represent mean ± SEM of four separate and independent experiments. ***indicates significant difference compared with the control at $p < 0.001$.

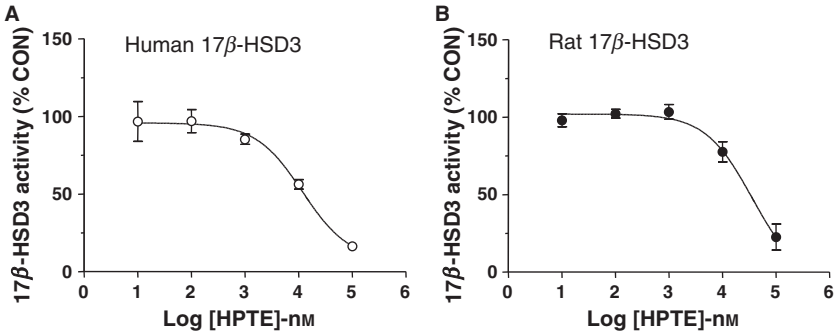

Figure 3 Inhibition of 17β-hydroxysteroid dehydrogenase (17β-HSD3) activity by 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in human (A) and rat (B) testis microsomes ($n = 4$). 17β-HSD3 activity was assessed by conversion of androstenedione (DIONE, 100 nM) to testosterone (T) in the presence or absence of different concentrations of HPTE. Conversion rate of control was 78.5 and 45.56% for the human and rat samples, respectively. IC_{50} values were calculated from these assays and are shown in Table 1 (mean ± SEM).

greater affinity for the oestrogen receptor (Bulger *et al.*, 1978a). Furthermore, at the 100 μM concentration, MXC did not affect either human or rat 17β-HSD3 activity. However, HPTE showed significant inhibition of 17β-HSD3 activity with IC_{50} of 12 μM for human 17β-HSD3 and 32 μM for the rat enzyme. Thus, it appears that MXC is metabolically activated for the suppression of both 3β-HSD and 17β-HSD3 activity.

MXC and HPTE inhibition of human and rat 3β-HSD were non-competitive for enzyme substrates. Therefore, we investigated whether MXC competes with the cofactor NAD^+ (for 3β-HSD) and examined the effects of different concentrations of cofactors in the presence of varying concentrations of the inhibitor (MXC or HPTE). The results indicated that MXC and HPTE were competitive with the NAD^+ cofactor in the inhibition of 3β-HSD. Thus, it is reasonable to infer that MXC and HPTE block binding of NAD^+ to the cofactor-binding site of 3β-HSD while not interfering with the PREG active site. Furthermore, present data showed that HPTE was non-competitive with the substrate DIONE for 17β-HSD3 enzyme inhibition, but was competitive with the NADPH cofactor in the same regard. Therefore, we submit that HPTE blocks binding of NADPH to the cofactor-binding site of 17β-HSD3 and does not compete for the DIONE substrate-binding site of the enzyme. As MXC and HPTE bind to cofactor-binding sites of 3β-HSD and 17β-HSD, it appears that inhibition caused by MXC or HPTE action is specific for hydroxysteroid dehydrogenases. Indeed, MXC was found to inhibit human 3β-HSD at 100 nM (Fig. 1), whereas it did not affect human 17β-HSD3 even at 100 μM (Table 1).

Given the requirement for steroidogenic enzyme activity for androgen biosynthesis, inhibition of 3β-HSD and 17β-HSD3 by the metabolite HPTE following exposure to MXC may be a contributing factor to its testicular toxicity. In this study, we demonstrated that concentrations as low as 10 nM of MXC or HPTE suppressed human 3β-HSD activity, although the IC_{50} were much higher in the micromolar range. Nevertheless, these observations are relevant to public health because MXC

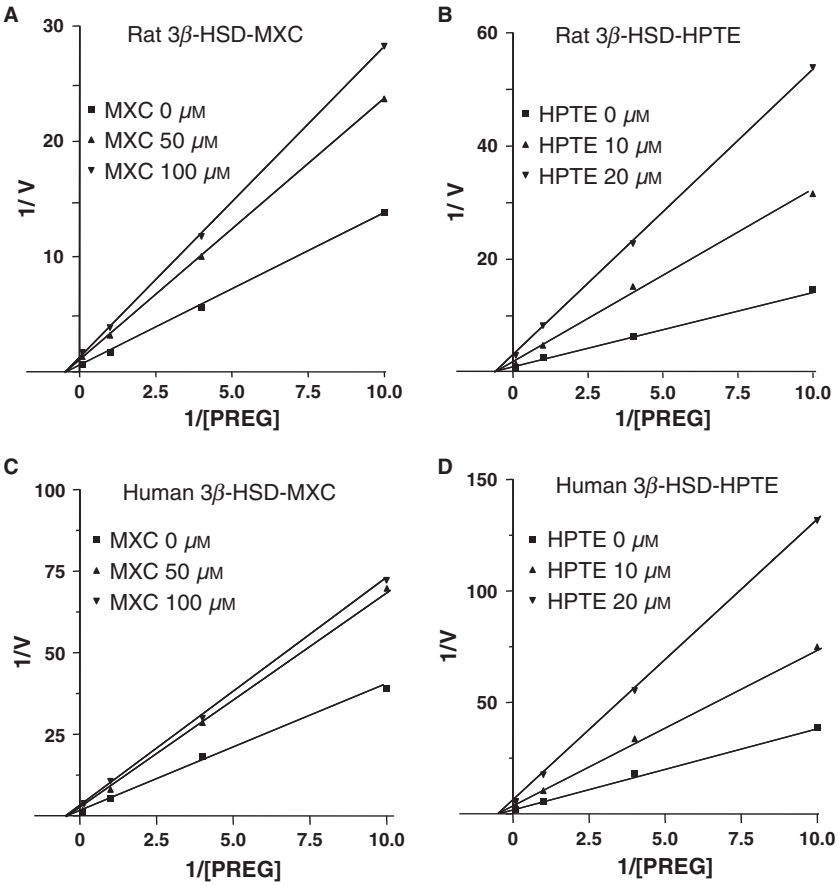

Figure 4 Lineweaver–Burk plot of human and rat testis microsomal 3β-hydroxysteroid dehydrogenase (3β-HSD) kinetics in the presence of methoxychlor (MXC) and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). 3β-HSD was measured by conversion of different concentrations of pregnenolone (PREG) to progesterone in 60 μg of protein for 30 min. Data represent mean ± SEM of four separate and independent experiments. (A) and (B) list the effects of MXC and HPTE on rat 3β-HSD activity; (C) and (D) list the effects of MXC and HPTE on human 3β-HSD activity.

occurs in the environment at comparable levels. For example, the level of MXC in fish can reach levels as high as 128 μg/kg (Kumar & Byrtus, 1993). Such concentrations of MXC or HPTE have the potential to affect 3β-HSD and 17β-HSD3 activity.

Importantly, there is evidence that exposure to MXC adversely affects male reproductive tract development in several animal models. For example, exposure of male rats to 100 or 200 mg/kg/day of MXC from postnatal day 21 into adulthood delayed preputial separation, reduced epididymal weight, seminal vesicle weight, sperm production and serum testosterone levels (Gray *et al.*, 1989). In addition, male rats exposed to 50 or 150 mg/kg/day of MXC in utero from gestational day 14 through postnatal day 7 and thereafter by oral gavage through postnatal day 42 exhibited delayed preputial separation, which signifies reduced testosterone levels and diminished androgen-stimulated action (Chapin *et al.*, 1997). Serum testosterone levels are dependent in part on Leydig cell steroidogenic enzyme activity, and testosterone is required

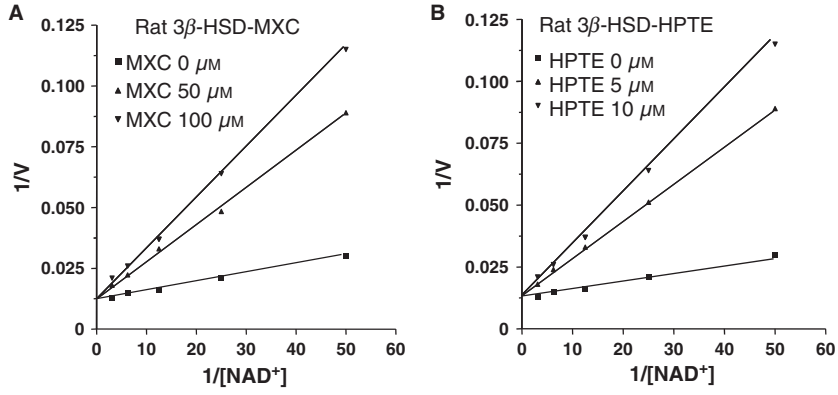

Figure 5 Lineweaver–Burk plots of rat testis microsomal 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in the presence of methoxychlor (MXC) or 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) plus NAD^+. 3β-HSD was measured by conversion of 0.2 μM of pregnenolone to progesterone in 60 μg of protein for 30 min in the presence of various concentrations of NAD^+. Data represent mean ± SEM of four separate and independent experiments. (A) and (B) list the effects of MXC and HPTE on 3β-HSD activity.

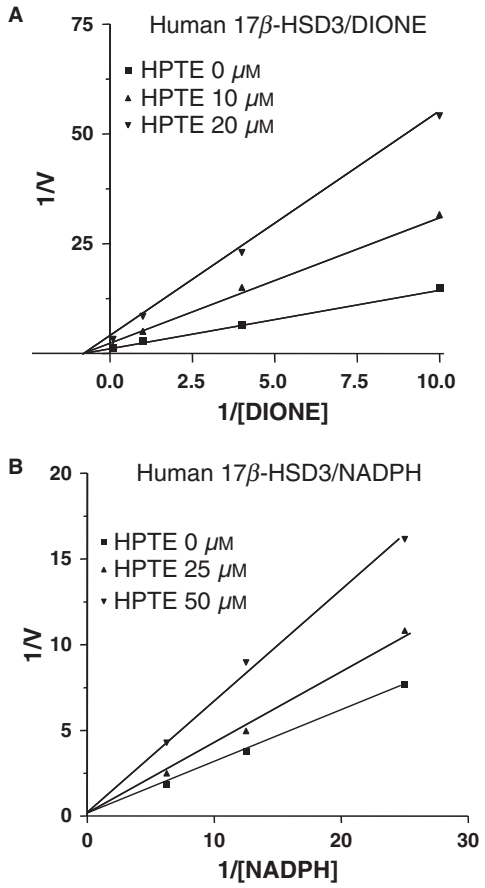

Figure 6 Lineweaver–Burk plot of human testis microsomal 17β-hydroxysteroid dehydrogenase (17β-HSD3) activity in the presence of 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). (A) Plot with different concentrations of androstenedione (DIONE) plus 0.2 mM of NADPH; (B) plot with different concentrations of NADPH plus 0.1 μM of DIONE. Data represent mean ± SEM of four separate and independent experiments.

for development of the male reproductive tract in the foetal period (Akingbemi & Hardy, 2001) as well as initiation and maintenance of male puberty and spermatogenesis in adulthood (Ge *et al.*, 2007). Therefore, inhibition of testicular steroidogenesis by MXC or its metabolite HPTE has adverse implications for male reproductive activity.

Previous studies demonstrated that HPTE regulated androgen biosynthesis by acting through oestrogen receptors (Akingbemi *et al.*, 2000). Indeed, HPTE was found to inhibit competitively, binding of [^{3}H]oestradiol to testicular oestrogen receptors at concentrations as low as 100 nM (Bulger *et al.*, 1978a), whereas the IC_{50} to displace oestradiol from a recombinant human oestrogen receptor-α was calculated at 750 nM (Bolger *et al.*, 1998). Although the IC_{50} to inhibit human 3β-HSD was 8.75 μM, which is about 10-fold less potent than its displacement of oestradiol from human oestrogen receptor-α, the minimal effective concentration of HPTE inhibiting human 3β-HSD was as low as 10 nM, indicating that inhibition of human 3β-HSD may be involved in its suppression of Leydig cell steroidogenesis. Although Akgul *et al.* (2008) demonstrated that HPTE significantly inhibited P450 cholesterol side-chain cleave enzyme directly at 50 nM and above through a non-ER-mediated action, our previous results indicated that HPTE action was ER-mediated (Akingbemi *et al.*, 2001). Thus, it will appear that HPTE action occurs by multiple mechanisms. There appears to be consensus that low concentrations of ER agonists generally act by receptor-mediated mechanisms and that the ER requires only between 0.1% and 10% receptor occupancy to become transcriptionally active (Nagel *et al.*, 1999). The IC_{50} for HPTE-induced ER-mediated activity was 1.11 μM (Akingbemi *et al.*, 2001), which is much lower than the 13.8 μM required for direct inhibitory action (present results). Thus, it is perhaps reasonable to infer that exposures to low concentrations of MXC and HPTE will cause ER-mediated activity, but that increasing exposure levels will additionally involve direct inhibition of enzyme activity.

This study demonstrated that HPTE was more potent than MXC to inhibit human and rat 3β-HSD and 17β-HSD3 activity. For example, the IC_{50} of HPTE-inhibiting human 3β-HSD and 17β-HSD3 were 8.3 and 12.1 μM, respectively, whereas MXC inhibited human 3β-HSD with an IC_{50} of 53.2 μM, and did not affect human 17β-HSD3 at concentrations as high as 100 μM (Table 1). This observation indicates that MXC is more potent after metabolic degradation to HPTE, and presumably exerts greater affinity for the ER. For example, MXC was found to bind human oestrogen receptor-α with an IC_{50} of 81–193 μM whereas the IC_{50} for HPTE action was calculated at 750 nM (Bolger *et al.*, 1998).

The results further showed that MXC is a non-competitive inhibitor of 3β-HSD, whereas its metabolite HPTE is a potent inhibitor of both 3β-HSD and 17β-HSD3. Many DDT isomers, including MXC, continue to be used for agricultural purposes and are present in the environment. Thus, these compounds pose a health risk not only to agricultural workers but also to the general population. The results presented herein identify additional mechanisms of action of MXC and HPTE in regulating androgen biosynthesis and reinforce previous observations showing that these compounds have the capacity to cause adverse biological effects affecting testis function. Identification of the mechanisms of action of hormonally active agents present in the environment is required to facilitate the process of risk assessment of the population.

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