Mechanism of Induction of Cytochrome P450 Enzymes by the Proestrogenic Endocrine Disruptor Pesticide—Methoxychlor: Interactions of Methoxychlor Metabolites with the Constitutive Androstane Receptor System

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ABSTRACT:

Methoxychlor, a structural analog of the DDT pesticide, was previously shown to induce rat hepatic CYP2B and -3A mRNAs and the corresponding proteins [J Biochem Mol Toxicol 1998;12:315-332]. Additionally, methoxychlor was found to activate the constitutive androstane receptor (CAR) system and induce CYP2B6 (J Biol Chem 1999;274:8043-8046), suggesting a mechanism for methoxychlor-mediated cytochrome P450 (P450) 2B induction. However, it has not been established whether CAR activation and P450 induction was due to methoxychlor per se and/or due to its metabolites. Also, a possible link between the estrogenic potency of methoxychlor metabolites and CAR activation or P450 induction was not investigated. The current study explores the ability of methoxychlor and its metabolites to activate CAR and whether their potency of CAR activation correlates with their respective estrogenicity. Methoxychlor and its metabolites (mono-OH-M [1,1,1-trichloro-2-(4-methoxyphenyl)-2-(4-hydroxyphenyl)ethane]; bis-OH-M [1,1,1-trichloro-2-(4-methoxyphenyl)-2-(3-hydroxy-4-methoxyphenyl)ethane]; ring-OH-M [1,1,1-trichloro-2-(4-methoxyphenyl)-2-(3-hydroxy-4-methoxyphenyl)ethane]; and tris-OH-M [1,1,1-trichloro-2-(4-methoxyphenyl)-2-(3,4-dihydroxyphenyl)ethane]) were found to be potent activators of CAR. Dose response curves indicated that tris-OH-M is a more potent CAR activator than methoxychlor, mono-OH-M, and bis-OH-M. Since tris-OH-M is a much weaker estrogen receptor-α agonist than mono-OH-M and bis-OH-M, it seems that estrogenicity is not a significant factor in CAR activation. These findings indicate that alteration of methoxychlor-benzene rings, i.e., generation of phenolic constituents, does not appreciably alter CAR activation and suggest that a common structural motif in the methoxychlor class of compounds controls CAR activation. Studies are needed to identify the structural motif necessary for CAR activation and CYP2B induction.

Methoxychlor, a structurally analogous to DDT, is used as a substitute for DDT, which has been banned in the industrially developed countries (Metcalf, 1976). The increased usage of methoxychlor is attributed to its facile biodegradability, its relatively short half-life in animals, and its low toxicity in mammals as compared with DDT (Metcalf, 1976; Kupfer, 1982). However, despite the favorable characteristics, there is considerable concern for the possible toxicity of methoxychlor in animals and humans. This concern stems from observations that the major methoxychlor metabolites exhibit estrogenic activity (Bulger et al., 1978, 1985; Kupfer and Bulger, 1980a,b; Osterhout et al., 1981; Kupfer, 1982) and that certain methoxychlor metabolites display antiandrogenic activity (Maness et al., 1998; Gaido et al., 2000). Additionally, methoxychlor elicits reproductive toxicity in mammals (Bal and Mungkorn, 1978; Cummings and Gray, 1989; Cummings and Laskey, 1993), apparently because of its hormonal activity. Consequently, methoxychlor has been undergoing extensive toxicology studies as a prototype endocrine disruptor (Chapin et al., 1997; Cummings, 1997).

Since DDT induces hepatic cytochrome P450 (P450) (CYP2B and CYP3A) enzymes in rats, mimicking phenobarbital (PB)-type inducers (Hart and Fouts, 1965; Lubet et al., 1992; Li et al., 1995) and because methoxychlor is a structural analog of DDT (Fig. 1A), it was assumed that methoxychlor will induce the same P450s as DDT. Indeed, the administration of methoxychlor to rats induced hepatic CYP2B and -3A enzymes (Li et al., 1995). Additionally, Northern blot analyses of the mRNAs encoding these enzymes and nuclear run-on experiments indicated that the induction was attributable to increased rate of transcription (Li and Kupfer, 1998).

The profile of the P450 isoforms induced by methoxychlor in rats suggested that methoxychlor is a PB-type inducer. Support for that notion resides in the demonstration that several PB-type inducers activate the artificially repressed nuclear receptor CAR system from HepG2 human hepatoma cell line and induce NR1 luciferase reporter activity (Sueyoshi et al., 1999). CAR is an orphan nuclear hormone
A receptor that is translocated from the cytosol to the nucleus when a suitable ligand is present (Kawamoto et al., 1999). Evidence that CAR can increase human P450 gene expression was obtained when it was shown that the 3α-androstenol-repressed CAR is activated by a variety of ligands, among these PB, methoxychlor, and o,p' -DDT, resulting in induction of CYP2B6 (Sueyoshi et al., 1999).

Whether the P450s’ induction in vivo (Li et al., 1995; Li and Kupfer, 1998) was mediated by methoxychlor per se or via methoxychlor metabolite(s) has not been hitherto addressed. Realizing that the examination of that question in vivo would have yielded only equivocal information, we explored that question in vitro by examining the metabolic scheme of methoxychlor. The combined hexane solution was washed with water until the pH was neutral. The hexane phase was evaporated to dryness under stream of N2. After a precubination for 2 min at 37°C, the reaction was initiated by the addition of a freshly prepared NADPH-regenerating system [glucose 6-phosphate (50 μmol); 50 μl (5 μmol) of magnesium chloride; microsomes (0.075 mg of protein); [14C]methoxychlor (12.5 nmol; 20,000 dpm) in 5 μl of ethanol; and H2O2, yielding a volume of 450 μl. (After a precubination for 2 min at 37°C the reaction was initiated by the addition of a freshly prepared NADPH-regenerating system [glucose 6-phosphate (5 μmol); NADPH (0.25 μmol); and glucose-6-phosphate dehydrogenase (1 IU) in 50 μl of phosphate buffer (pH 7.4, 5 μmol)], and the vials were incubated under air atmosphere with shaking (37°C for 60 min). The reaction was terminated with 2 ml of ethanol, and the resulting aqueous-alcoholic mixture was centrifuged. The supernatant was removed and the vial was rinsed with 2 ml of ethanol. The combined supernatants were evaporated under a stream of N2.

Animals and Treatment. Male Sprague-Dawley CD rats (90–100 g), from Charles River Breeding Laboratory (Wilmington, MA), were housed under controlled temperature (22°C) and light (12-h light/dark cycle; lights off at 7:00 PM). Rats were injected i.p. with PB (37.5 mg/kg in 0.2 ml of water twice daily for 4 days), and liver microsomes were prepared 12 h after the last injection (see below).

Chemicals. TCPOBOP [1,4-bis(2-(3,5-dichloropyridyloxy)]benzene] was synthesized by using the method of Kende et al. (1985). 3α-Androstenol was obtained from Steraloids (Newport, RI). NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and EDTA were from Sigma Chemical Co. (St. Louis, MO). Ultima-gold scintillation fluid was from Packard (Downers Grove, IL). All other chemicals were of reagent grade quality and were used without purification. Methoxychlor (laboratory grade) was from Chem Service (West Chester, PA) and was purified (see below). Diphenyl-DDT (98% pure) was from Aldrich (Milwaukee, WI).

Preparation of Hepatic Microsomes. Rat liver microsomes were prepared by homogenization of the livers (usually four to eight livers) in 0.25 M sucrose (5 ml/g of liver) using a Potter-Elvehjem (Teflon pestle) homogenizer (Eberbach Corp., Ann Arbor, MI) followed by differential centrifugation (Burstein and Kupfer, 1971; Dehal and Kupfer, 1996). The microsomal pellet (sedimented at 105,000g) was suspended in 1.15% aqueous KCl, followed by centrifugation (105,000g for 1 h). The microsomal pellet was overlaid with fresh 1.15% aqueous KCl solution and stored at −70°C until use.

Purification of Methoxychlor. Methoxychlor was dissolved in ethanol, and the solution was made alkaline (pH 11–12) with 0.1 N sodium hydroxide. The alkalinized solution was immediately extracted with hexane (3 × 5 ml). The combined hexane solution was washed with water until the pH was neutral. The hexane phase was evaporated to dryness under stream of N2 gas, and the residue was crystallized from ethanol. The purified methoxychlor exhibited a single UV absorbing spot on normal phase TLC (fluorescent indicator) in a system of chloroform/acetone (9:1, v/v). Purity was at least 98%.

Preparation of Methoxychlor Metabolites. Ring-OH-M. Rat liver microsomal pellet was thawed and suspended in fresh 1.15% KCl solution, and appropriate aliquots were incubated with [14C]methoxychlor (Dehal and Kupfer, 1994). The incubations were in 7-ml glass vials containing the following constituents: 300 μl (30 μmol) of sodium phosphate buffer (pH 7.4) containing EDTA (0.5 μmol); 50 μl (5 μmol) of magnesium chloride; microsomes (0.075 mg of protein); [14C]methoxychlor (12.5 nmol; 20,000 dpm) in 5 μl of ethanol; and H2O2, yielding a volume of 450 μl. After a precubination for 2 min at 37°C, the reaction was initiated by the addition of a freshly prepared NADPH-regenerating system [glucose 6-phosphate (5 μmol); NADPH (0.25 μmol); and glucose-6-phosphate dehydrogenase (1 IU) in 50 μl of phosphate buffer (pH 7.4, 5 μmol)], and the vials were incubated under air atmosphere with shaking (37°C for 60 min). The reaction was terminated with 2 ml of ethanol, and the resulting aqueous-alcoholic mixture was centrifuged. The supernatant was removed and the vial was rinsed with 2 ml of ethanol. The combined supernatants were evaporated under a stream of N2. The concentrated sample was spotted on a reverse phase TLC and developed in a solvent system of methanol/water/acetic acid (75:24:1, v/v/v). The TLC plate was radio-scanned with Bioscan system 2000 (Washington, DC). The radiolabeled methoxychlor metabolites that migrated with a Rf corresponding to authentic samples were scrapped off the plate, and the metabolites were eluted with ethanol (3 × 2 ml); the eluants were evaporated to dryness under a stream of N2. The residue dissolved in 0.5 ml of ethanol was analyzed using the above chromatographic conditions. The ring-OH-M displayed analytical characteristics (chromatographic and spectral) identical to those reported by Dehal and Kupfer (1994). Purity was ~96%.

Preparation of mono-OH-M. Mono-OH-M, custom synthesized from methoxychlor by GL Synthesis (Worcester, MA), was spotted on a normal phase TLC, and the plate was subjected to chromatography in a solvent system of chloroform/acetone (9:1, v/v). A control lane of the plate was visualized under UV light, and the band region corresponding to mono-OH-M in an adjacent lane (not exposed to UV) was scrapped off and eluted with ethanol (3 × 3 ml). The combined ethanolic eluate was evaporated to dryness under N2, and the residue was taken up in 0.5 ml of ethanol. The purified mono-OH-M yielded a single spot on TLC (purity at least 94%).

Tris-OH-M. Tris-OH-M was synthesized by Chem Syn (Lenexa, KS) and used without purification. In high-performance liquid chromatography, the compound showed a single UV peak (purity ~98%).

Cells and Transfection. (NR1)1-tk-luciferase reporter plasmid which contains quintuple NR1 sequence in PB-responsive enhancer of CYP2B6 gene was previously constructed (Kawamoto et al., 1999). Both transfection of g2car3 cells by calcium phosphate precipitation and reporter gene assay, using Dual-Luciferase Reporter Assay System (Promega, Madison, WI), were performed as previously described (Sueyoshi et al., 1999). After allowing transfection to proceed for 18 h, the cells were further treated with each chemical for 24 h and
Results and Discussion

Methoxychlor is metabolized by the P450 system in liver microsomes of animals and humans via O-demethylation and hydroxylation reactions (Fig. 1B). The pathway involves mono- and bis-demethylation of methoxychlor (yielding mono-OH-M and bis-OH-M metabolites, respectively) followed by ortho-hydroxylation resulting in the catechol tris-OH-M (Kupfer et al., 1990). The methoxychlor demethylation reactions in human liver are catalyzed by several CYP2C isoforms and by CYP1A2 (Stresser and Kupfer, 1998a), and the subsequent hydroxylation reaction is primarily by CYP3A4 (Stresser and Kupfer, 1997, 1998b). However, there is an additional pathway (Fig. 1B), involving monohydroxylation of methoxychlor (ortho to methoxy), forming the ring-OH-M metabolite (Dehal and Kupfer, 1994). This product was observed in incubations with low microsomal protein concentrations and brief incubation time. However, incubations of methoxychlor with 1 mg/ml of microsomal protein did not yield ring-OH-M, but instead provided tris-OH-M. We found that ring-hydroxylation of methoxychlor is catalyzed by CYP2B1 (rat) and CYP2B6 (human), and this reaction is followed by dual demethylations, yielding tris-OH-M (Stresser et al., 1996). Thus, the major and essentially final product of both pathways is tris-OH-M. Occasionally, a minor (more polar) product has been observed and is tentatively referred to as tetra-OH-M (Fig. 1B).

To determine whether methoxychlor and/or methoxychlor metabolites are responsible for P450 induction, we used the in vitro CAR system. In that system, we quantify luciferase reporter activity from (NR1)\(_5\)-tk-luciferase construct in g2car3 cells treated with or without chemicals. Because constitutive activity of CAR hinders inducibility by chemicals, the baseline of CAR activation is suppressed by adding androstenol (8 \(\mu\)M), as previously reported (Sueyoshi et al., 1999), prior to adding the various methoxychlor metabolites (at 50 \(\mu\)M). The activity of methoxychlor and metabolites was compared with that of TCPOBOP (Fig. 2). Results indicate that methoxychlor and each of the metabolites exhibited pronounced induction of CAR. However, because of paucity in the availability of ring-OH-M metabolite, that compound was tested at a single dose of 3.90 \(\mu\)M, yielding a relative luciferase activity of 2.91; this value indicates a somewhat lower degree of induction than that obtained with tris-OH-M, which at 3.13 \(\mu\)M yielded a relative luciferase activity of 2.76. When dose response curves were generated with methoxychlor, mono-OH-M, bis-OH-M, and tris-OH-M (Fig. 3, A and B), mono-OH-M exhibited similar potency to methoxychlor. Tris-OH-M appeared to be the most potent inducer among the metabolites, followed by bis-OH-M and mono-OH-M (Fig. 3B). These results demonstrate that methoxychlor metabolism may not be crucial for significant activation of CAR; nevertheless, metabolism of methoxychlor seems to enhance the level of induction. Hence, it appears that the basic methoxychlor structure, i.e., the trichloroethane side chain and the two benzene rings on the C2 of the ethane moiety, are adequate structural features for induction. This is substantiated by the observation that a methoxychlor analog, lacking ring substituents and referred to as diphenyl-DDT (Fig. 1A), activated CAR (i.e., induced the NR1 reporter luciferase activity) almost with similar potency to methoxychlor (not shown). This finding further demonstrated that functional groups (methoxyls or hydroxyls) are not essential for activity. However, there is a caveat to this conclusion, since the g2car3 cell line derived from HepG2 may not be totally metabolically inert toward methoxychlor. Furthermore, there is a possibility that the HepG2 cell line hydroxylates the aromatic ring of diphenyl-DDT, and that the latter product activates CAR. Thus, further studies would be needed to substantiate our claim that methoxychlor and diphenyl-DDT could also be active per se, albeit with somewhat weaker potency. Consequently, a structure-activity relationship would be necessary (in metabolically inert cell line) to ascertain which structural motif in methoxychlor is absolutely necessary for CAR activation and whether that structural feature correlates with in vivo induction of CYP2B and CYP3A enzymes.

Tris-OH-M exhibits weak estrogen agonist activity, being much less active in the transactivation assay of the estrogen receptor-\(\alpha\) (ER\(\alpha\)) than either bis-OH-M or mono-OH-M (Gaido et al., 2000). However, by contrast, tris-OH-M appears to be a more potent inducer of CAR than either bis-OH-M or mono-OH-M. Thus, it seems that the degree of estrogenicity of methoxychlor derivatives has little or no bearing on their respective CAR activation. Nevertheless, since the modulation of the ER receptor by TCDD has been attributed to “cross-talk” between the ligand (TCDD)-bound aryl hydrocarbon receptor and ER (Nguyen et al., 1999; Wormke et al., 2000), it would be of interest to assess whether there is a potential interaction between the ER and CAR systems in response to the various methoxychlor metabolites. Such a study will require the use of a cell line containing transfected CAR and ER\(\alpha\) or ER\(\beta\) genes in a potential cross-talk proximity (preferentially in a cell line lacking significant P450 activity and thus having no metabolic capability) and examining the effects on CAR activation of compounds that modulate the ER, e.g., TCDD.
of ERβ (e.g., bis-OH-M) (Gaido et al., 2000) should be tested in that system. Last, it would be of interest to determine whether direct binding of the ligands, such as methoxychlor metabolites, to CAR is necessary for CAR activation and whether the affinity of the ligands for CAR correlates with their relative potency of CAR activation.

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