EXTRAHEPATIC METABOLISM OF CARBAMATE AND ORGANOPHOSPHATE THIOETHER COMPOUNDS BY THE FLAVIN-CONTAINING MONOOXYGENASE AND CYTOCHROME P450 SYSTEMS

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ABSTRACT:
The cytochrome P450 (P450) and flavin-containing monooxygenase (FMO) enzymes are the major oxidative enzymes in phase I metabolism. Many organophosphate and carbamate thyoether compounds are excellent substrates for these enzymes. Stereoselective sulfoxidation of fenthion and methiocarb by human liver, kidney, and microsomes was investigated. A high level of stereoselectivity in the formation of fenthion (+)-sulfoxide was observed in kidney and intestinal microsomes. This activity was not inhibited by the P450 inhibitor 1-aminobenzotriazole but was dramatically reduced following mild heat treatment. In liver, fenthion was metabolized to its sulfoxide in a nonstereoselective manner, and the activity was sensitive to both 1-aminobenzotriazole and heat treatment. The carbamate pesticide methiocarb also was sulfoxidated with a high degree of stereoselectivity in human kidney microsomes. Human liver microsomes formed both stereoisomers in equal amounts. Sulfoxide formation in kidney was not inhibited by 1-aminobenzotriazole but was abolished in liver microsomes. Formation of methiocarb sulfoxides was not observed in intestinal microsomes. The relative contribution of FMO1 and FMO3 to the sulfoxidation of carbofuranon, demeton-O, ethiofencarb, fono fos, and methiocarb also was investigated by using baculovirus-expressed recombinant proteins. FMO1 showed the highest catalytic activity for all pesticides. This study indicates that FMO1 may have a bigger role in extrahepatic metabolism than previously thought.

The P450 and FMO systems are multigene families involved in the oxidative metabolism of a wide range of xenobiotics. The P450 family is a superfamily of monooxygenases with at least 57 isoforms identified in humans (http://drnelson.utmcm.edu/hum.html). They are heme-containing proteins that exist in a multienzyme system that also includes NADPH-cytochrome P450 reductase and cytochrome b5. The FMO family is genetically less diverse than the P450s, with only five functional genes (FMO1–5) currently identified in humans (Lawton et al., 1994). Although both systems are dependent on NADPH as a cofactor, the FMO family accepts reducing equivalents directly from NADPH without the need for a NADPH reductase (Ziegler and Mitchell, 1972).

The P450 and FMO families have partially overlapping substrate specificities and may or may not form the same metabolites. Differences in stereoselectivity also are observed between the two groups as well as between the isoforms (Levi and Hodgson, 1988; Park et al., 1993). The mammalian P450s are catalytically more diverse than the FMOs and are associated with the metabolism of important endogenous compounds, such as arachidonic acid and testosterone, as well as bioactivation and detoxification of numerous xenobiotics. The FMO-catalyzed pathway is generally thought to be a detoxification process in which lipophilic compounds are converted to more polar compounds by introducing molecular oxygen.

P450 enzymes are involved in both oxidative desulfuration of the phosphorothionate to form the oxon and oxidation of the thiocarbonate group to the corresponding sulfoxide and sulfone of various organophosphates (Dauterman, 1971). The FMOs are generally limited to the formation of sulfoxides (Hajjar and Hodgson, 1980). Many of the organophosphates are potent inhibitors of acetylcholinesterase, and oxidative metabolism may further bioactivate these compounds to inhibitors that are manyfold more potent than the parent compound (Fukuto, 1990). A recent comprehensive study on oxidative organophosphate metabolism by P450 and FMO isoforms found sulfoxidation of several thiocarbonate pesticides in human liver microsomes to be mainly P450-driven (85–90%), with the remainder accounted for by FMO (Usmani et al., 2004). Among the 16 P450 isoforms and 3 FMO isoforms studied, CYP3A4, the CYP2C family, and FMO1 had the highest turnover numbers. Previous studies in our laboratory have observed that FMO1 catalyzed the sulfoxidation of carbamate and organophosphate pesticides much more efficiently than FMO3 (Schlenk et al., 2002; Furnes and Schlenk, 2004). In adult humans, FMO3 is the major liver isoform, and FMO1 primarily is found in kidney and intestines (Dolphin et al., 1996; Overby et al., 1997; Yeung et al., 2000). Thus, the contribution of the FMO system to hepatic organophosphate metabolism is thought to be modest. However, current knowledge on the contribution of oxidative systems, especially FMO, to extrahepatic organophosphate metabolism is lacking.

In this study we report on the sulfoxidation of five pesticides,
carbophenothion (S-4-chlorophenylthiomethyl O,O-dimethyl phosphorothioate), demeton-O (O,O-diethyl O-2-ethylthioethyl phosphorothioate), ethiofencarb (α-ethylthio-o-tolyl methylcarbamate), fonofos [O-ethyl S-phenyl (RS)-ethyphosphonodithioate], and methiocarb (4-methylthio-3,5-xylyl methylcarbamate) (Fig. 1) by the major human FMO isofoms FMO1 and FMO3. These pesticides have diverse structural features making them good candidates for probing FMO activity. Due to the lack of information on extrahepatic organophosphate and carbamate thioether metabolism, we also investigated the contribution of the P450 and FMO systems to stereoselective sulfonidation of fenthion (O,O-dimethyl O-4-methylthio-o-tolyl phosphorothioate) and methiocarb in human liver, kidney, and intestinal microsomes. Our studies establish a role of FMO1 in the metabolism of these pesticides in both kidney and intestinal microsomes. The role of the FMO system in oxidative pesticide metabolism, particularly in extrahepatic tissues, may be greater than previously thought.

Materials and Methods

Chemicals. Carbophenothion, demeton-O, ethiofencarb, fenthion, fenthion sulfoxide, fonofos, methiocarb, and methiocarb sulfoxide were purchased from Chem Service Inc. (West Chester, PA). All solvents used for HPLC were of HPLC grade and purchased from Fisher Scientific Co. (Pittsburgh, PA). If not specified, all other reagents were also purchased from Fisher Scientific or Sigma-Aldrich (St. Louis, MO).

Human FMO Isoforms and Human Liver, Kidney, and Intestinal Microsomes. FMO1 and FMO3 were expressed as recombinant proteins using a baculovirus system as previously described (Furnes and Schlenk, 2004) and quantified by an HPLC flavin assay (Lang et al., 1998). Pooled human liver, kidney, and intestinal microsomes were purchased from In Vitro Technologies, Inc. (Baltimore, MD). They were not characterized with regard to P450 and FMO protein content.

In Vitro Metabolic Assays. Kinetic parameters for FMO1 and FMO3 sulfonation of carbophenothion, demeton-O, ethiofencarb, fonofos, and methiocarb were determined by measuring NADPH oxidation at 340 nm. A 1-ml reaction volume consisting of 50 mM glycine buffer, pH 9.0, 0.2 mM NADPH, 3.3 mM MgCl2, and 0.5 to 0.8 nmol FMO1 or FMO3 was preincubated for 3 min before adding substrate. Reactions were carried out in triplicate. An NADPH extinction coefficient (εmax) of 6220 M⁻¹ was used to calculate FMO1- and FMO3-dependent NADPH oxidation.

General incubation reactions for liver, kidney, and intestinal microsomes were carried out using a 0.25-ml total volume containing 0.5 mg of microsomes, 100 μM potassium phosphate buffer (pH 7.4), 5.0 mM MgCl2, 1 mM NADPH, and 250 μM substrate. Fenthion and methiocarb were dissolved in a methanol solution, and the final concentration of methanol in the reaction was less than 1%. The contribution of P450 and FMO to sulfonidation of fenthion and methiocarb was studied by selectively inhibiting each enzyme system. FMOs were inhibited by heat treatment at 50°C for 90 s. P450 isoforms were inhibited by 1 mM 1-aminobenzotriazole (1-ABT) added 10 min before the substrate. 1-ABT is a mechanism-based nonspecific P450 inhibitor. Stereoselectivity of recombinant FMO1 and FMO3 with regard to methiocarb sulfonation was determined at a single concentration of 250 μM. The reactions were terminated after 15 min by the addition of 0.75 ml of dichloromethane. After addition of internal standard [(R)-methyl p-tolyl sulfoxide], the samples were vortexed for 60 s and centrifuged for 5 min at 3000 g. The organic layer was removed under a stream of nitrogen and the metabolites were reconstituted in

![Image of structures of organophosphate and carbamate thioether compounds](image)

FIG. 1. Structures of organophosphate and carbamate thioether compounds used in the investigation.

Values are reported as mean ± S.E.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FMO1</th>
<th>FMO3</th>
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<tbody>
<tr>
<td></td>
<td>V (100 μM)</td>
<td>Vmax</td>
</tr>
<tr>
<td></td>
<td>(nmol/min/nmol)</td>
<td>μM</td>
</tr>
<tr>
<td>Carbo phenothion</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>Demeton-O</td>
<td>18.0 ± 1.3</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td>Ethiofencarb</td>
<td>14.9 ± 1.5</td>
<td>30 ± 1.3</td>
</tr>
<tr>
<td>Fonofos</td>
<td>5.1 ± 0.7</td>
<td>-</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>15.1 ± 0.9</td>
<td>28 ± 1.4</td>
</tr>
</tbody>
</table>

N.D., not detected.

*−*, kinetic parameters were not determined due to low activity and solubility.

TABLE 1

Substrate specificity of expressed FMO1 and FMO3
carbophenothion and fonofos prevented determination of activities. Limited catalytic velocities and solubility of these conditions, the fenthion (+)- and (−)-sulfoxides had retention times of 13.6 and 18.4 min, respectively (6:3:1 mobile phase). The unknown fenthion sulfoxide concentrations were determined by extrapolating against a standard curve. Methiocarb sulfoxides had retention times of 14.6 and 15.4 min (designated peak 1 and peak 2, respectively) and were quantified against a standard curve of methiocarb sulfoxide. We were not able to obtain the pure methiocarb stereoisomers for determination of optical activity. Detection sensitivity of fenthion sulfoxide and methiocarb sulfoxide was 100 μl of hexane/isopropanol/dichloromethane (5:3:2). Reactions were carried out in duplicate.

**HPLC Analysis of Metabolites.** Fenthion and methiocarb sulfoxides were separated using a Regis Technologies, Inc. (Morton Grove, IL) Whelk-01 10/100 (R,R) chiral column with a mobile phase consisting of hexane/isopropanol/dichloromethane in a 6:3:1 or a 5:3:2 ratio at a flow rate of 1 ml/min. The sulfoxides were detected at a wavelength of 254 nm. Under these conditions, the fenthion (+)- and (−)-sulfoxides had retention times of 13.6 and 18.4 min, respectively (6:3:1 mobile phase). The unknown fenthion sulfoxide concentrations were determined by extrapolating against a standard curve with known amounts of fenthion sulfoxide. Methiocarb sulfoxides had retention times of 14.6 and 15.4 min (designated peak 1 and peak 2, respectively) and were quantified against a standard curve of methiocarb sulfoxide. We were not able to obtain the pure methiocarb stereoisomers for determination of optical activity. Detection sensitivity of fenthion sulfoxide and methiocarb sulfoxide was 100 μl of 2.5 and 7 μM standards, respectively. Recovery of internal standard was typically greater than 90%.

**Kinetic Analysis.** Kinetic parameters were determined using Prism 3.0 (GraphPad Software Inc., San Diego, CA). A one-site binding model was used to establish $K_m$ and $V_{max}$. Results are expressed as means ± S.E. Data were analyzed by ANOVA with Dunnett’s multiple range test to compare catalytic activities. $P < 0.05$ was the accepted level of significance.

**Results and Discussion**

The P450 and FMO families are the main oxidative enzymes involved in organophosphate and carbamate sulfoxidation in humans (Dauterman, 1971; Tynes and Hodgson, 1985). Organophosphate and carbamate pesticides remain heavily used due to their relatively low nontarget toxicity and rapid degradation after application. For most individuals, exposure to these compounds occurs from residential pesticide usage or in the form of residues from various food groups such as dairy products, vegetables, and fruits (Krieger, 1995; Jaga and Dharmami, 2003). The first barrier for such compounds to enter systemic circulation is transportation and metabolism across the intestinal wall. There is limited knowledge on the extrahepatic metabolism, especially within the intestine, of organophosphate pesticides in humans.

FMO1 showed higher turnover numbers than FMO3 for all pesticides studied (Table 1). Demeton-O, ethiofencarb, and methiocarb displayed Michaelis-Menten kinetics, and $V_{max}$ and $K_m$ were determined for all compounds. Limited catalytic velocities and solubility of carbophenothion and fonofos prevented determination of $K_m$ and $V_{max}$. FMO1 stereoselectively formed the methiocarb sulfoxide peak 2 enantiomer (>98%), whereas FMO3 did not form sufficient sulfoxides to determine stereoselectivity. FMO1 has previously been found to metabolize fenthion with much higher turnover numbers than FMO3 (Furnes and Schlenk, 2004) and numerous P450 isoforms (Kitamura et al., 2003a,b). In rat liver, FMO1 is the major oxidase involved in fenthion oxidation, and recombinant human FMO1 is catalytically more active than human and rat P450s (CYP1A1, CYP2E1).

TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Fenthion (250 μM) Ratio of (+)-Sulfoxide to (−)-Sulfoxide Formation</th>
<th>Methiocarb (250 μM) Ratio of Peak 1 to Peak 2 Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+1-ABT 50°C</td>
<td>+1-ABT 50°C</td>
</tr>
<tr>
<td>Kidney</td>
<td>90:10 96:4 60:40</td>
<td>2:98* 2:98* N.D.</td>
</tr>
<tr>
<td>Intestine</td>
<td>91:9 92:8 56:44</td>
<td>N.D. N.D. N.D.</td>
</tr>
</tbody>
</table>

N.D., sulfoxides not detected or at too low amounts to establish stereoselectivity.

$^*$ Peak 1 was not detected. With the sensitivity of the HPLC system, >98% of the sulfoxide formed was peak 2.

**FIG. 2.** HPLC chromatograms of products obtained from the incubation of fenthion with human liver, kidney, and intestinal microsomes. Fenthion sulfoxide standard (100 μl of 30 μM solution) was analyzed on a chiral HPLC column (A). Fenthion sulfoxides were eluted with a 6:3:1 hexane/isopropanol/dichloromethane mobile phase with retention times of 13.6 and 18.4 min for the (+)- and (−)-sulfoxide, respectively. Liver (B), kidney (C), and intestinal (D) microsomes were incubated with a 250 μM concentration of fenthion as described under Materials and Methods. Metabolites were analyzed by HPLC under the same conditions as the pure fenthion sulfoxide standard. The peak at 11.4 min was the internal standard. The additional peaks observed in the HPLC chromatograms were not affected by adding 1-ABT or heat treatment.
CYP1A2, CYP2B6, CYP2C9, and CYP3A4). FMO1 is also very specific for the formation of the (+)-sulfoxide, whereas FMO3 forms the (+)-sulfoxide in 75% excess. On the other hand, the carbamate pesticide methiocarb is highly metabolized by both P450 and FMO isoforms (Buronfosse et al., 1995; Usmani et al., 2004). Based on this knowledge, we chose to use fenthion and methiocarb as model compounds for extrahepatic metabolism.

In kidney and intestinal microsomes, formation of the fenthion (+)-sulfoxide was almost exclusive and not affected by the P450 inhibitor, 1-ABT (Table 2; Figs. 2 and 3). Mild heat treatment, known to significantly reduce FMO activity, did strongly inhibit formation of the (+)-sulfoxide, indicating that this is an FMO1-catalyzed reaction. In liver, turnover numbers were relatively low and only weakly affected by both inhibiting P450 and FMO isoforms, suggesting a role for both systems in fenthion sulfoxidation. The P450 system seems to be responsible for methiocarb sulfoxidation in human liver since preincubation with 1-ABT reduced formation of both sulfoxides significantly (Fig. 4). The stereoselective formation of only one of the methiocarb sulfoxides (peak 2) in kidney microsomes was not inhibited by 1-ABT, indicating that FMO1 was likely involved. The lack of methiocarb sulfoxidation in intestinal microsomes is believed to be due to sensitivity of the HPLC system rather than absence of metabolism. Formation of the peak 2 sulfoxide was observed but was below a 3:1 signal to noise ratio and thus not quantified. These data indicate that compounds such as methiocarb that are highly metabolized by the P450 system in liver have the potential for undergoing FMO-mediated metabolism in other tissues.

The overlap between the P450 isoforms expressed in intestines and the P450 isoforms that show high catalytic turnover numbers for organophosphate pesticides strongly suggests that the P450 system is important in intestinal metabolism of most organophosphates (Usmani et al., 2004). However, for compounds such as fenthion, FMO1 metabolism may be dominant. Human FMO1 has also demonstrated effective turnover numbers with commonly used drugs (Mushiroda et al., 2000; Stevens et al., 2003a), and this might affect bioavailability.

To summarize, our data provide strong evidence that FMO1 is an important extrahepatic pathway in humans and, in particular, it confirms a role for FMO1 in the metabolism of carbamate and organophosphate thioethers in the intestine and kidney.

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References


FIG. 3. Sulfoxidation of fenthion by human liver, kidney, and intestinal microsomes. Human liver, kidney, and intestinal microsomes were incubated with 250 µM fenthion as described under Materials and Methods. The metabolites were separated on a chiral HPLC column with retention times of 14.6 (peak 1) and 15.4 (peak 2) min. The peak 2 methiocarb sulfoxide was observed in intestinal microsomes but at too low levels to quantify (3:1 signal to noise ratio). Recombinant FMO1 stereoselectively formed the peak 2 sulfoxide (data not shown). Values are reported as nmol/min/mg ± S.E.

FIG. 4. Sulfoxidation of methiocarb by human liver, kidney, and intestinal microsomes. Human liver, kidney, and intestinal microsomes were incubated with 250 µM methiocarb as described under Materials and Methods. The metabolites were separated on a chiral HPLC column with retention times of 14.6 (peak 1) and 15.4 (peak 2) min. The peak 2 methiocarb sulfoxide was observed in intestinal microsomes but at too low levels to quantify (3:1 signal to noise ratio). Recombinant FMO1 stereoselectively formed the peak 2 sulfoxide (data not shown). Values are reported as nmol/min/mg ± S.E.


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