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Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases

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Abbreviations:
CDMB, 2-chloro-3,4-dimethoxybenzil
hCE-1, human carboxylesterase 1
hCE-2, human carboxylesterase 2
rCE, rabbit carboxylesterase
alpha-Cypermethrin, αRS-cyano-(3-phenoxyphenyl)methyl (1RS)-*cis*-3-(2,2-dichlorovinyl)-2, 2-dimethyl-cyclopropanecarboxylate
deltamethrin, αS-cyano-(3-phenoxyphenyl)methyl (1R)-*cis*-3-(2,2-dibromovinyl)-2, 2-dimethyl-cyclopropanecarboxylate
trans-permethrin, 3-phenoxbenzyl (1RS)-*trans*-3-(2,2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate
cis-permethrin, 3-phenoxbenzyl (1RS)-*cis*-3-(2,2-dichlorovinyl)-2, 2-dimethyl-cyclopropanecarboxylate

ABSTRACT

Pyrethroid chemicals are attractive alternatives to the organophosphates (OPs) because of their selective toxicity against pests rather than mammals. The carboxylesterases (CEs) are hepatic enzymes that metabolize ester-containing xenobiotics such as pyrethroids. The primary aim of this study was to gain insight into the catalytic properties of the CE enzymes in humans that metabolize pyrethroids, while a secondary aim was to investigate pyrethroid metabolism using CEs from other mammalian species. Pure human CEs (hCE-1 and hCE-2), a rabbit CE (rCE), and two rat CEs (Hydrolases A and B) were used to study the hydrolytic metabolism of the following pyrethroids: 1R *trans*-resmethrin (bioresmethrin), 1RS *trans*-permethrin, and 1RS *cis*-permethrin. hCE-1 and hCE-2 hydrolyzed *trans*-permethrin 8- and 28-fold more efficiently than *cis*-permethrin (when k_{cat}/K_m values were compared), respectively. In contrast, hydrolysis of bioresmethrin was catalyzed efficiently by hCE-1, but not by hCE-2. The kinetic parameters for the pure rat and rabbit CEs were qualitatively similar to the human CEs when hydrolysis rates of the investigated pyrethroids were evaluated. Further, a comparison of pyrethroid hydrolysis by hepatic microsomes from rats, mice, and humans indicated that the rates for each compound were similar between species, which further supports the use of rodent models for pyrethroid metabolism studies. An eight-fold range in hydrolytic rates for 11 individual human liver samples toward *trans*-permethrin was also found, although this variability was not related to the levels of hCE-1 protein in each sample. We also determined that the CE inhibitor 2-chloro-3,4-dimethoxybenzil blocked hCE-2-catalyzed *trans*-permethrin hydrolysis 36 times more potently than hCE-1. Thus, this inhibitor will be useful in future studies that examine CE-mediated metabolism of pyrethroids. While there are likely other esterases in human liver that hydrolyze pyrethroids, the results of this study clearly demonstrate that hCE-1 and hCE-2 are human pyrethroid-hydrolyzing CEs.

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bioresmethrin,
(5-benzyl-3-furyl)methyl
(1R)-*trans*-2,2-dimethyl-3-
(2-methylprop-
1-enyl)-cyclopropanecarboxylate
4-MUBA, 4-methyl
umbelliferyl acetate
p-NPA
para-nitrophenyl acetate
p-NPV, *para*-nitrophenyl valerate
p-NPB, *para*-nitrophenyl valerate
p-NPB, *para*-nitrophenyl butyrate
3PBAlc, 3-phenoxybenzyl alcohol
Cl_2CA, *cis*/*trans*-3-(2',2'-dichlorovinyl)-
2,2-dimethylcyclopropane
carboxylic acid
3PBAld, 3-phenoxybenzaldehyde
hAChE, human
acetylcholinesterase
hBChE, human
butyrylcholinesterase

1. Introduction

The quantities of pyrethroid insecticides used in agriculture are rapidly increasing [1] due in part to their limited toxicity to mammals and their good spectrum of activity against crop damaging pests. However, adverse effects in humans may still occur following exposure to these compounds, with neurotoxicity being the primary side effect following acute exposure [2]. Furthermore, studies have shown that pyrethroids can elicit neurodevelopmental deficits following chronic low doses [3]. This class of compounds may also affect levels of xenobiotic metabolizing enzymes in the liver. For example, permethrin was shown to act as a phenobarbital-like inducer of cytochrome P450 2B isozymes in hepatocytes [4,5], while cypermethrin and fenvalerate can directly activate the pregnane *X* receptor (PXR) [6]. Thus, pyrethroids may potentially elicit ''pesticide-pesticide'' and/or ''pesticide-drug'' interactions during co-exposures to pharmaceutical compounds and other pesticides.

The parent pyrethroid molecule is largely responsible for the toxic effects elicited by these compounds and metabolism is considered a detoxification mechanism [1]. Metabolic studies conducted in rodents demonstrated that pyrethroids are degraded rapidly to polar metabolites by oxidative and hydrolytic enzymes (for review, see [7]). In vitro studies using rodent liver microsomes elucidated the panel of metabolites formed in these systems [8]. However, the precise nature of the metabolic enzymes responsible for pyrethroid biotransformation is presently unknown except in broad categories, e.g., the cytochrome P450 and carboxylesterase (CE) families. These enzyme families are expressed abundantly in the mammalian liver [9] and utilized by insects to gain resistance against insecticides [10].

The CEs (EC 3.1.1.1) are members of the serine hydrolase superfamily of esterases and catalyze the hydrolysis of esters, amides, and thioesters [11]. CEs possess a triad of amino acid residues (serine, histidine, and glutamic acid) that are essential for activity; mutation of any one of these residues results in a non-functional enzyme [12]. CEs have broad substrate specificity and are abundantly expressed in the mammalian liver. Several highly homologous CE isozymes that exhibit overlapping substrate specificities have been identified in rodents [13–17].

Two major carboxylesterases have been identified in human liver and are termed hCE-1 and hCE-2 [18,19], while a third hepatic CE (hCES3) has been recently isolated and is expressed at lower levels than the other CEs [20]. The amino acid sequences of hCE-1 and hCE-2 differ from each other by 48% and because of their divergent homology are placed into separate CE classes (class 1 for hCE-1 and class 2 for hCE-2). The most abundant CE expressed in the human intestine is termed hiCE and it differs from hepatic hCE-2 by a six amino acid truncation at the *N*-terminus; however, they are functionally equivalent enzymes [12,21]. The specificity of each human CE isozyme with respect to pyrethroid hydrolysis reactions has not been adequately studied to this point.

The primary aim of this study was to examine whether the human carboxylesterases hCE-1 and hCE-2 are pyrethroid-hydrolyzing esterases and to determine if these enzymes will be useful biomarkers of susceptibility in populations that are exposed to these xenobiotics. A secondary aim was to investigate pyrethroid metabolism using CEs from other mammalian species. The expression of hCE-1 and hCE-2 in *Spodoptera frugiperda* has enabled their isolation in yields necessary for biochemical and structural analyses [22,23]. In this study, we have used recombinant hCE-1, hCE-2, rabbit CE (rCE), and two pure rat CEs (Hydrolases A and B) to study the hydrolytic metabolism of the type I pyrethroids: bioresmethrin, 1*RS trans*-permethrin, and 1*RS cis*-permethrin, and (to a limited extent) the type II pyrethroids: alpha-cypermethrin and deltamethrin (see Fig. 1 for structures and stereochemistry of these compounds). Type II pyrethroids possess a cyano group at the alpha carbon adjacent to the esterified oxygen atom; type I pyrethroids lack this cyano group [7]. Our results

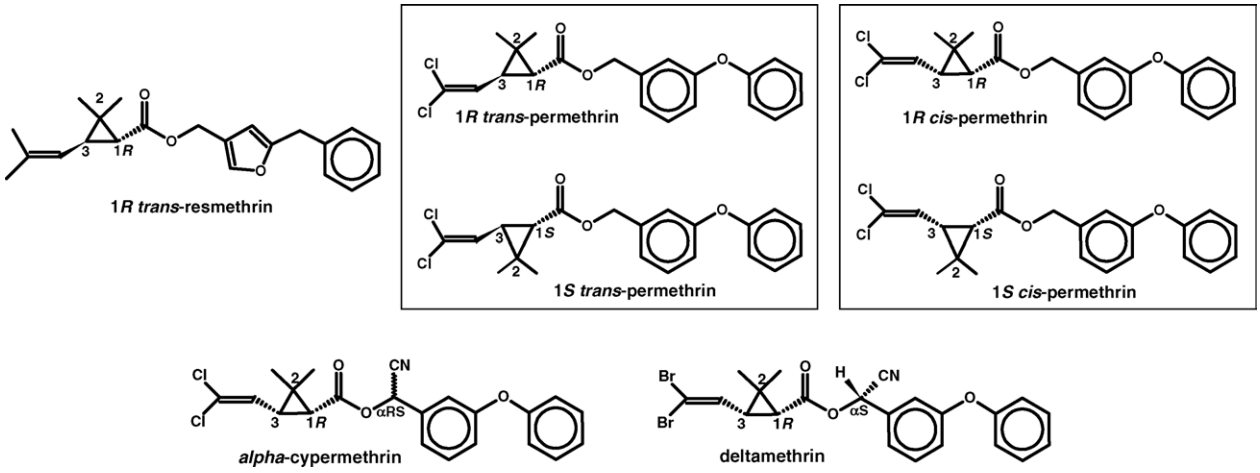

Fig. 1 – Structures of pyrethroids used in this study. The stereocenters are denoted as *R/S* and *cis/trans*.

shed light on the specificity and activities of two human CE isozymes toward a series of pyrethroid commonly used in agricultural practices, and define the kinetic parameters for hydrolytic enzymes largely responsible for detoxifying these compounds in vivo.

2. Materials and methods

2.1. Chemicals

Type I pyrethroids: 1*RS trans*-permethrin, 1*RS cis*-permethrin, and 1*R trans*-resmethrin (bioresmethrin); type II pyrethroids: alpha-cypermethrin and deltamethrin were obtained from Chem Service (West Chester, PA). 1*RS*/1*R* and *cis*/*trans* indicate the absolute stereochemistry at the C_1 and C_3 atoms of the cyclopropane ring of the pyrethroid, respectively (see Fig. 1); however, the designation 1*RS* is omitted for clarity in the text. The following authentic standards of pyrethroid metabolites were purchased from Sigma-Aldrich (St. Louis, MO): 3-phenoxybenzyl alcohol (3PBAlc), *cis*/*trans*-3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (1:1 mixture of *cis* and *trans* isomers) [also called *cis*/*trans*-dichlorochrysanthemic acid (Cl_2CA)], 1*R trans*-chrysanthemic acid (CA), and 3-phenoxybenzaldehyde (3PBAld). 3-(4-Methoxy)-phenoxybenzaldehyde, *para*-nitrophenyl acetate (p-NPA), *para*-nitrophenyl butyrate (*p*-NPB), 4-methylumbelliferyl acetate (4-MUBA), benzil (diphenylethane-l,2-dione), and 2-chloro-3,4-dimethoxybenzil (CDMB) were obtained and used without further purification from Sigma. *para*-Nitrophenyl valerate (*p*-NPV) was synthesized and kindly provided by Dr. Howard Chambers (Department of Entomology, Mississippi State University). Tetraethylpyrophosphate was purchased from Chem Service and 1,2-bis(3,5-difluorophenyl)-ethane-1,2-dione was synthesized as described previously [24].

2.2. Enzymes

Recombinant CE enzymes [rabbit CE (rCE); rabbit CESA (rCESA, serine residue at position 221 in the active site was mutated to an alanine residue); human CE-1 (hCE-1); and human intestinal CE (hiCE), also called hCE-2, which is the terminology used for this isozyme in this report] were expressed in *S. frugiperda* using baculovirus and purified to homogeneity as previously described [22]. Recombinant human acetylcholinesterase (hAChE) and human butrylcholinesterase (hBChE) were obtained from Sigma. Rat CEs termed Hydrolases A and B [16] were purified to homogeneity from male Sprague–Dawley rat liver by the procedure of Sanghani et al. [17] with slight modification. This entailed removal of a 80 kDa impurity that was present in the Hydrolase A preparation by anion exchange chromatography.

2.3. Antibodies

Polyclonal antibodies raised against the following CEs: human CE-1, rat hydrolase A (also called p*I* 6.1 or RH1 [15,16]), and rat hydrolase B (also called RL1; [16]) were kindly provided by Dr. M. Hosokawa (Chiba University, Japan). The protein content of the anti-hCEl antibodies was determined by the bicinchoninic acid (BCA) reagent assay (Pierce, Rockford, IL) using control rabbit IgG (Sigma) as the protein calibrant.

2.4. Liver microsomes

Liver microsomes and cytosols were prepared from male Sprague–Dawley rats and male BL6 mice by differential centrifugation methods [25]. The microsomes were washed in 100 mM potassium pyrophosphate buffer (pH 7.4) containing 1.0 mM EDTA and 20 μM butylated hydroxytoluene, recentrifuged (100,000 × *g*, 60 min), and the final microsomal pellet was resuspended in 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol. Eleven individual human liver microsomal samples and a pooled human liver microsomal sample were obtained from BD Biosciences (Woburn, MA). Donor information is provided in Supplementary Table SI. The protein content of the recombinant enzymes and the subcellular fractions was estimated using the BCA reagent assay with bovine serum albumin (Pierce) as the protein calibrant.

2.5. Pyrethroid hydrolysis reactions

2.5.1. Carboxylesterases (human, rabbit, and rat)

Hydrolysis of pyrethroids by CEs were performed in reaction volumes between 100 and 250 μL. Varying amounts of pyrethroid (5–100 μM) were pre-incubated for 5 min in 50 mM Tris–HCl buffer (pH 7.4) at 37 °C. The hydrolytic reactions were initiated by addition of the CE (between 1 and 10 μg per reaction) and the reaction was allowed to proceed for 10–15 min at 37 °C. Preliminary experiments showed that the production of hydrolysis products was linear up to 60 min. The reactions were quenched by the addition of an equal volume of ice-cold acetonitrile containing 10 μM of internal standard [3-(4-methoxy)-phenoxybenzaldehyde]. The samples were centrifuged for 5 min at 16,100 × *g* (4 °C) and an aliquot was analyzed by HPLC to quantify the hydrolysis products. Non-enzymatic controls were also performed and found to have negligible rates. CE reactions at each substrate concentration were performed in duplicate and three separate preparations of CEs were utilized.

2.5.2. Mammalian liver microsomes

Hydrolytic reactions catalyzed by rat, mouse, and human liver microsomal samples were performed in a total volume of 250 μL. Variable amounts of pyrethroid substrate (5–100 μM) were pre-incubated in 50 mM Tris–HCl buffer (pH 7.4) for 5 min (37 °C). Reactions were started by the addition of liver microsomes (final protein concentration, 0.5 mg/mL). After 15 min incubation, the reactions were quenched by the addition of an equal volume of ice-cold acetonitrile containing internal standard. After centrifugation, hydrolysis products were quantitated by HPLC. The stability of the permethrin hydrolysis product 3PBAlc was examined (at a concentration of 50 μM) in rat liver microsomes (0.5 mg protein/mL) over a period of 30 min at 37 °C. The levels of 3PBAlc remained steady throughout the 30 min incubation period, thus no significant oxidation of the alcohol was apparent.

Control experiments to inhibit serine hydrolase activities were also conducted using rat and human hepatic microsomes. Hepatic microsomes (0.5 mg/mL) were preincubated for 10 min with 120 μM tetraethylpyrophosphate (at 37 °C) prior to addition of the pyrethroid substrate. All other reaction conditions were the same as described above and products were analyzed by HPLC.

2.5.3. HPLC analysis

HPLC-UV analysis of pyrethroid hydrolytic products was performed on a Surveyor LC system (Thermo Electron, San Jose, CA) using a reversed-phase HPLC column (2.1 mm × 100 mm, C18, Thermo Electron). The mobile phases used were solvent A (1:1, v/v, water:acetonitrile containing 0.1%, v/v acetic acid) and solvent B (100% acetonitrile containing 0.1%, v/v acetic acid). Following injection of the sample onto the column, the analytes were eluted with the following gradient program: 0 min (100% A, 0% B), 6 min (100% A, 0% B), 20 min (50% A, 50% B), 25 min (50% A, 50% B), and 30 min (100% A, 0% B), at a flow rate of 0.2 mL/min. Products were detected at 230 nm. Calibration standards for the hydrolysis products were routinely run using an internal standard [3-(4-methoxy)-phenoxybenzaldehyde] and peak area ratios were plotted against the quantities of the analyte injected on-column.

For the *cis*- and *trans*-permethrin isomers, quantification of hydrolysis was based on the production of the alcohol product 3PBAlc. For bioresmethrin, hydrolysis rates were based on production of the carboxylic acid *trans*-CA. For alpha-cypermethrin and deltamethrin, hydrolysis rates were based on the production of *cis*-Cl_2CA and 3PBAld, respectively.

2.6. Spectrophotometric assay for esterase activity

The intrinsic esterase activities of the recombinant CE and mammalian liver microsomes were routinely assayed by measuring the production of *p*-nitrophenol liberated from *p*-NPV, *p*-NPA, or *p*-NPB at 400 nm on a spectrophotometer [26]. An extinction coefficient of 13 cm^{-1} mM^{-1} [16] was used to convert the slopes of each activity curve to specific activities. All enzymatic reaction rates were corrected by the non-enzymatic activities.

2.7. Immunoblotting of human liver microsomes

Human liver microsomal samples were analyzed by SDS-PAGE by standard procedures. After electrophoresis, the proteins were transferred to polyvinyldifluoride membranes and probed with rabbit anti-human CE-1 polyclonal antibody. Immunocomplexes were localized with a horse radish peroxidase-conjugated goat anti-rabbit secondary antibody and the SuperSignal West Pico Chemiluminescent substrate (Pierce). The chemiluminescent signal was captured using a digital camera (Alpha Innotech gel documentation system). The resulting digitized bands were quantified using NIH Image J software (v.l.33u).

2.8. Inhibition of carboxylesterases

Previous studies showed that hCE-2 was more sensitive than hCE-1 to the inhibitory effects of the compound CDMB [24]. Thus, we used this inhibitor to determine its ability to block the hydrolysis of *p*-NPV and *trans*-permethrin (or the indicated pyrethroid) using recombinant CE enzyme or hepatic microsomes.

Increasing concentrations of CDMB (ranging from 0.001 to 100 μM) were preincubated with 50 μM of pyrethroid or 500 μM of *p*-NPV for 5 min at 37 °C in 50 mM Tris–HC1 buffer (pH 7.4) before addition of CE enzyme or hepatic microsomes. *p*-NPV hydrolysis in the presence of CDMB was monitored spectrophotometrically for 2–5 min, while pyrethroid hydrolysis reactions proceeded for 15 min before being terminated; the pyrethroid reaction products were subsequently analyzed by HPLC as described above. The inhibition data were fitted to one of six equations describing competitive, partially competitive, non-competitive, partially non-competitive, mixed, or uncompetitive inhibition as described previously [28,29].

2.9. In-gel hydrolysis assays

Human liver microsomal samples were examined by native PAGE using 10% polyacrylamide mini-gels (Bio-Rad) followed by in-gel hydrolysis assay with 4-MUBA [27]. Following

electrophoresis, the gel was placed in a solution of 100 μM 4-MUBA in 0.1 M potassium phosphate buffer (pH 6.5) and gently rocked for 15 min at room temperature. The gel was then immediately placed on a UV transilluminator plate (302 nm) and the resulting fluorescence signals were quantified by densitometry.

2.10. Immunoprecipitation of hCE-1 in human liver microsomes by anti-hCE-1 antibody

The effect of immunoprecipitating hCE-1 on the hydrolysis of *trans*-permethrin by human liver microsomes was investigated. A pooled sample of human liver microsomes was solubilized with 0.5% (w/v) Brij78 (Acros, Pittsburgh, PA) in 10 mM Tris–HCl buffer (pH 7.4) for 1 h at 4 °C with gentle shaking. After centrifugation (16,100 × *g*, 4 °C, 5 min), aliquots of the supernatant were incubated with various amounts of rabbit anti-human CE1 polyclonal antibody (0–500 μg) overnight at 4 °C in 10 mM Tris–HCl buffer (pH 7.4). The total amount of antibody in each sample was adjusted to 500 μg with control rabbit IgG (Sigma). After 18 h, samples were centrifuged and aliquots of the supernatants (which contained ~200 μg microsomal protein) were incubated with 50 μM *trans*-permethrin for 15 min at 37 °C. The reactions were terminated with equal volumes of cold acetonitrile and analyzed by HPLC.

Pooled human liver microsomal samples that had been preincubated with anti-hCE-1 antibody were also assayed using the substrate *p*-NPA. The microsomal proteins were incubated with 500 μM substrate and the production of *p*-nitrophenol was monitored as indicated above.

2.11. Kinetic analysis and statistics

Non-linear regression of substrate concentration versus reaction velocity curves were analyzed using SigmaPlot v. 8.02 software by fitting experimental data to the Michaelis–Menten equation. Each substrate concentration in the kinetic experiments was evaluated in duplicate. Three separate preparations of each recombinant enzyme were utilized and the kinetic parameters were averaged and S.E.M. calculated. The specific activity data obtained for hepatic microsomes using standard substrates are reported as the mean (±S.D.) of three replicates.

3. Results

3.1. Pyrethroid hydrolysis reactions catalyzed by carboxylesterases

Recombinant CEs (i.e., rCE, hCE-1, and hCE-2) were expressed and purified from *S. frugiperda* as described previously [22]. The enzymes were tested for activity with three standard *para*-nitrophenyl esters of increasing acyl chain length (acetate, butyrate, and valerate) (Table 1). Each CE had maximal activity with the substrate *p*-NPB and hence, these enzymes appeared to favor a substrate acyl chain length of four carbons (butyrate) over chain lengths of two (acetate) or five (valerate). More importantly, rCE was found to have higher specific activities than either human CE isozyme, consistent with previous reports [24,30]. Thus, we used rCE to develop an assay to measure pyrethroid hydrolysis products.

In order to estimate steady-state kinetic parameters of CE-catalyzed pyrethroid hydrolysis reactions, an HPLC method was developed to quantify the levels of hydrolytic products. We initially used *trans*-permethrin and rCE to develop HPLC conditions. *trans*-Permethrin is hydrolyzed by CEs to 3-phenoxybenzyl alcohol (3-PBAlc) and *trans*-dichlorochrysanthemic acid (Cl_2CA) (see Fig. 2 for chemical reaction). HPLC chromatograms of the hydrolytic products of *trans*-permethrin following incubation with rCE is presented in Fig. 3A and a time-course for the reaction is shown in Fig. 3B. Hydrolysis of pyrethroid substrates by recombinant CEs increased in a time-dependent manner. Routinely, 15 min incubation periods were chosen for subsequent kinetic assays since less than 10% of the pyrethroid substrate was consumed during this time-frame. In contrast to wild-type rCE, the catalytically inactive mutant rCESA enzyme did not hydrolyse *trans*-permethrin because an alanine residue replaced the nucleophilic serine at position 221 of rCE (see Fig. 3B).

A comparison of the hydrolytic rates for types I and II pyrethroids catalyzed by rCE (Fig. 4) demonstrated that the type II compounds alpha-cypermethrin and deltamethrin were hydrolyzed more slowly than the type I compounds. The hydrolysis rate of bioresmethrin was 30-fold faster than alpha-cypermethrin, which had the slowest rate. Within the series of type I compounds, the rate of hydrolysis catalyzed by rCE was bioresmethrin > *trans*-permethrin > *cis*-permethrin (Fig. 4). Because the type II pyrethroids were not hydrolyzed effectively by rCE, we focused on studying the kinetic parameters of the type I compounds by human and other mammalian CEs (Table 2).

The substrate concentration versus reaction velocity plots for each pyrethroid/CE combination typically appeared hyperbolic, which is consistent with Michaelis–Menten kinetics. The kinetic parameters estimated for each recombinant CE (rCE, hCE-1, and hCE-2) and three type I pyrethroids are listed in Table 2. In general, bioresmethrin was rapidly hydrolyzed by all CE enzymes with the notable exception of hCE-2, which did not possess any detectable hydrolytic activity toward this compound (see Table 2). Thus, different CE isozymes within humans can exhibit markedly different specificities for the same pyrethroid substrate. In contrast, *trans*-permethrin was

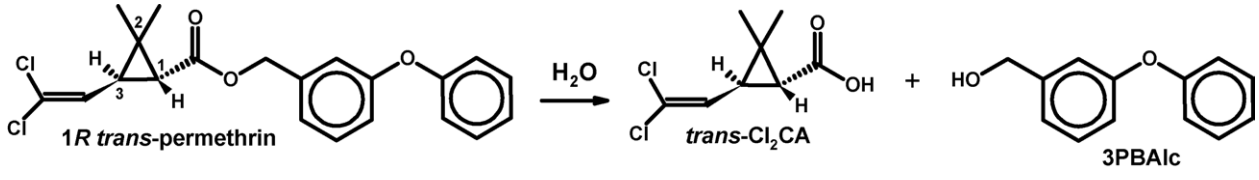

Fig. 2 – Hydrolysis of *trans*-permethrin to *trans*-dichlorochrysanthemic acid (Cl_2CA) and 3-phenoxybenzyl alcohol (3PBAlc).

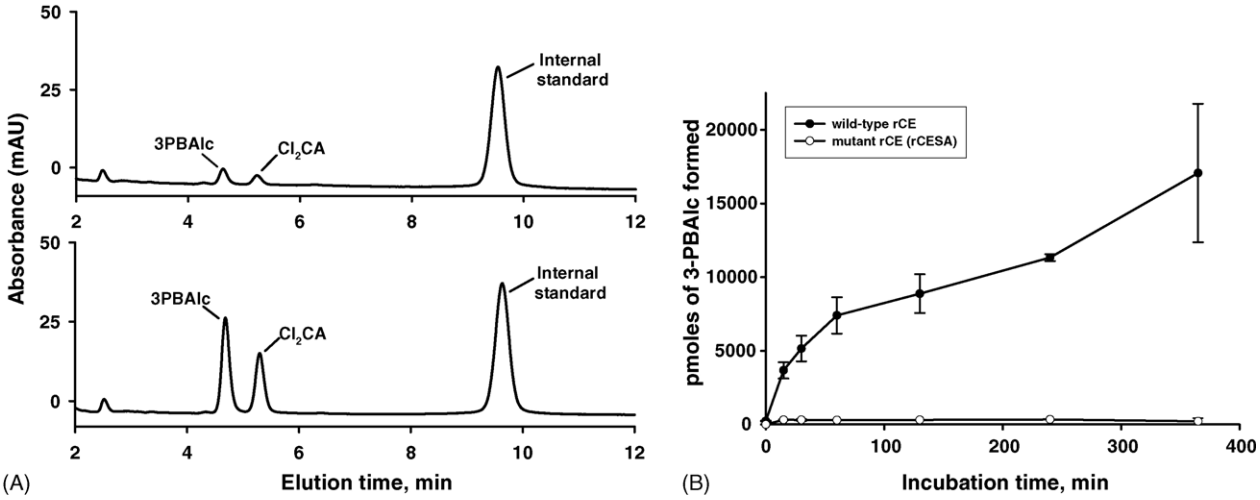

Fig. 3 – Hydrolysis of *trans*-permethrin by recombinant CEs. (A) HPLC-UV analysis of the products of the hydrolytic reaction catalyzed by rabbit CE. The top chromatogram is the analysis of a reaction with 5 μM *trans*-permethrin; the bottom chromatogram is the analysis of a reaction with 50 μM *trans*-permethrin. The hydrolysis products of *trans*-permethrin are denoted as 3PBAlc and *trans*-Cl_2CA; their structures are given in Fig. 2. The internal standard is 3-(4-methoxy)-phenoxybenzaldehyde. (B) Plot of the time-course of hydrolysis of *trans*-permethrin (50 μM) by wild-type and mutant rabbit CEs.

hydrolyzed more efficiently than *cis*-permethrin by both hCE-1 and hCE-2 (8- and 28-fold, respectively, when k_{cat}/K_m values are compared; Table 2). The *cis*- versus *trans*-difference in hydrolysis rates observed is consistent with literature reports that have shown *trans*-isomers of pyrethroids to be cleaved faster than *cis*-isomers by CEs [31–33]. In contrast to bioresmethrin, which was not hydrolyzed by hCE-2, *trans*-permethrin was cleaved efficiently by hCE-2 (k_{cat}/K_m = 195.8 min^{-1} mM^{-1}). Thus, it is likely both hCE-1 and hCE-2 have roles in the in vivo metabolism of *trans*-permethrin since their k_{cat}/K_m values are of the same magnitude (see Table 2) and both isozymes are expressed at relatively high levels in liver [37].

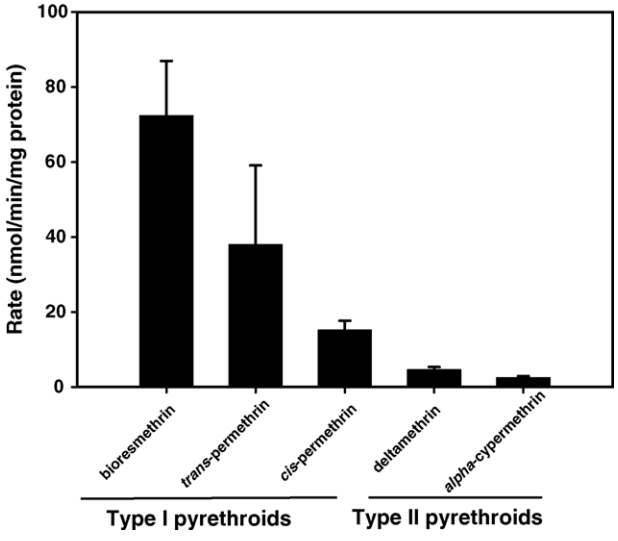

Fig. 4 – Comparison of rates of pyrethroid hydrolysis catalyzed by rabbit carboxylesterase. Rates were estimated using 50 μM of each pyrethroid substrate. Each value is the average ± S.E.M (n = 2–3 determinations).

The hydrolytic activities of the related human enzymes AChE and BChE toward *trans*-permethrin were also investigated to determine if these enzymes play a role in pyrethroid metabolism. Little hydrolytic activity was noted for these enzymes when compared to the CEs (estimated k_{cat} values for hAChE and hBChE were 0.008 and 0.002 min^{-1}, respectively). Thus, these enzymes do not appear to contribute to the metabolism of pyrethroids.

We also examined two rat CEs known as Hydrolases A and B, which are the two most abundant rat hepatic CEs [16], for their ability to hydrolyze *cis*- and *trans*-permethrin (Table 3). Hydrolases A and B share greater than 95 and 80% amino acid sequence homology, respectively, with the rabbit CE [11]. We found that *cis*-permethrin has a low K_m for both rat CEs and low turnover numbers (Table 3), which is generally similar to the kinetic parameters for rabbit and human CEs with *cis*-permethrin (Table 2). Furthermore, the rat CEs also exhibited high affinity (low K_ms) and high turnover numbers when *trans*-permethrin was the substrate, similar to the situation with human and rabbit CEs. Thus, kinetic parameters for the rat CEs were qualitatively similar to the rabbit and human CEs when hydrolysis of the permethrin isomers was evaluated.

3.2. *Pyrethroid hydrolysis reactions catalyzed by mammalian liver microsomes*

Comparison of the hydrolytic metabolism of bioresmethrin, *trans*-permethrin, *cis*-permethrin, and alpha-cypermethrin by hepatic microsomes derived from mouse, rat, and human liver (a pool of 25 individuals) demonstrated that these species exhibit similar kinetics (see Fig. 5A and Table 4). The rates of pyrethroid hydrolysis followed the compound rank order: bioresmethrin > *trans*-permethrin ≫ alpha-cypermethrin

Table 1 – Specific activities of recombinant CEs toward standard ester substrates[a]

Enzyme	*p*-Nitrophenyl acetate (*p*-NPA) (μmol/min/mg)	*p*-Nitrophenyl butyrate (*p*-NPB) (μmol/min/mg)	*p*-Nitrophenyl valerate (*p*-NPV) (μmol/min/mg)
rCE	86.41 ± 37.52	374.05 ± 13.02	125.98 ± 53.31
hCE-1	36.14 ± 5.68	293.10 ± 12.07	113.17 ± 15.77
hCE-2	19.05 ± 8.88	315.20 ± 38.99	90.06 ± 50.53

[a] Specific activities are the mean (±S.E.M.) obtained from pooled data of three separate experiments for each recombinant CE (*p*-NPA and *p*-NPV) (each experiment was performed in triplicate). *p*-NPB activity values are from one experiment for each CE performed in triplicate. The concentration of each substrate was 500 μM.

Table 2 – Steady-state kinetic parameters of recombinant carboxylesterases toward type I pyrethroids[a]

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Bioresmethrin			
rCE	25.74 ± 8.44	6.58 ± 1.32	256
hCE-1	6.72 ± 1.49	2.04 ± 0.10	304
hCE-2	n.d.[b]	n.d.[b]	–
1*RS cis*-permethrin			
rCE	1.96 ± 0.01	0.95 ± 0.15	480
hCE-1	9.80 ± 10.27	0.17 ± 0.04	17
hCE-2	7.57 ± 2.23	0.05 ± 0.003	7
1*RS trans*-permethrin			
rCE	16.70 ± 4.88	3.05 ± 1.68	183
hCE-1	23.77 ± 10.04	3.39 ± 1.58	143
hCE-2	8.63 ± 3.53	1.69 ± 0.58	196

[a] Values are the mean (±S.E.M.) obtained from two to three experiments (each substrate concentration in a kinetic experiment was assayed in duplicate).
[b] n.d., kinetic parameters could not be obtained because the levels of hydrolysis products were below the HPLC detection limits.

Table 3 – Steady-state kinetic parameters of rat hydrolases A and B toward *cis*- and *trans*-permethrin

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
cis-Permethrin			
Hydrolase A	1.93 ± 0.96	0.24 ± 0.01	124
Hydrolase B	4.96 ± 1.83	0.32 ± 0.01	64.5
trans-Permethrin			
Hydrolase A	6.74 ± 5.39	2.29 ± 0.38	340
Hydrolase B	9.80 ± 4.64	1.49 ± 0.27	152

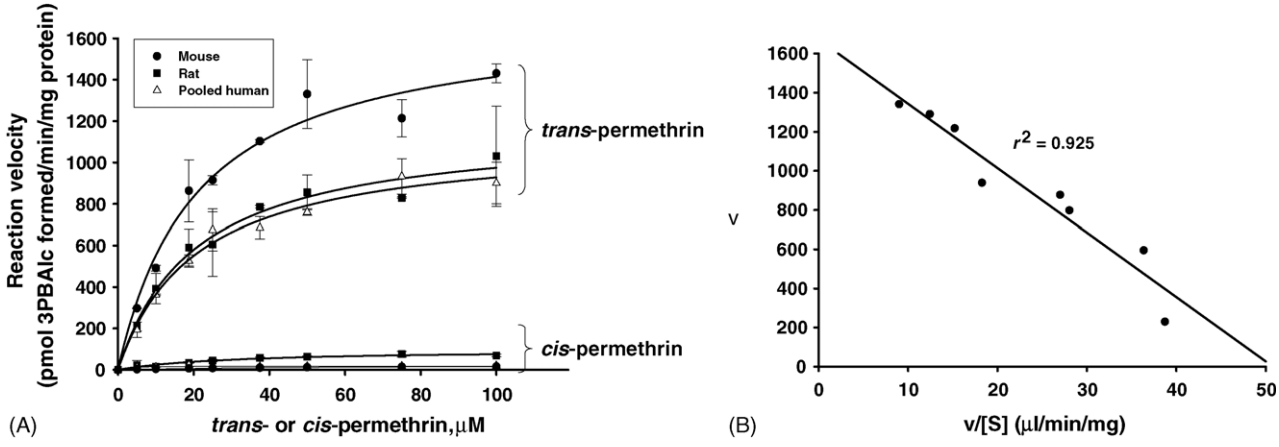

Fig. 5 – Hydrolysis kinetics of *trans*- and *cis*-permethrin using mouse, rat, and pooled human liver microsomes. (A) Substrate concentration-velocity data were fit to the Michaelis–Menten equation. The levels of 3-phenoxybenzyl alcohol (3PBAlc) formed were quantified. (B) Transformation of the pooled human liver kinetic data in (A) by Eadie–Hofstee plot. The units for the ordinate (V) are pmol 3PBAlc formed/min/mg protein.

Table 4 – Kinetic parameters of pyrethroid hydrolysis catalyzed by hepatic microsomes[a]

Species	Bioresmethrin		*trans*-Permethrin		*cis*-Permethrin		alpha-Cypermethrin	
	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
Rat[b]	46.60 ± 13.90	4.54 ± 0.44	19.16 ± 3.24	1.34 ± 0.27	30.78 ± 9.25	0.10 ± 0.01	13.16 ± 6.45	0.51 ± 0.07
Mouse	53.98 ± 14.63	6.62 ± 0.90	20.95 ± 3.78	1.71 ± 0.11	41.50 ± 25.55	0.02 ± 0.006	6.62 ± 4.70	0.09 ± 0.01
Human (pooled)	33.39 ± 7.49	2.57 ± 0.24	20.66 ± 2.95	1.12 ± 0.05	2.49 ± 2.14	0.02 ± 0.002	9.22 ± 3.27	0.14 ± 0.01

[a] Values (±S.E.) were obtained by non-linear regression of kinetic plots.
[b] Parameters for rat liver microsomes and bioresmethrin and *trans*-permethrin are the average (±S.D.) of three independent kinetic experiments.

> *cis*-permethrin for hepatic microsomes from all species. For each type I compound, the difference in V_{max} values between the rodent and human microsomes was less than three-fold and no marked differences were observed for the K_m values, except the low K_m for *cis*-permethrin and human liver microsomes (Table 4). Eadie–Hofstee kinetic plots obtained for each pyrethroid and microsomal sample were linear (see Fig. 5B), which suggested the following interpretations: (i) a single CE in the human, rat, and mouse hepatic microsomes was responsible for the bulk of the pyrethroid hydrolytic metabolism; or, alternatively, (ii) several different CEs exist in hepatic microsomes that hydrolyze these compounds and each possess similar kinetic parameters. The latter possibility is the likely scenario since multiple CEs, which possess pyrethroid-hydrolyzing activities, are known to be expressed in mammalian liver [26,32–34].

Pre-incubation of rat or human (pooled) liver microsomes with the general esterase inhibitor tetraethyl pyrophosphate (TEPP) for 10 min before addition of *trans*-permethrin completely inhibited the pyrethroid hydrolyzing activity of hepatic microsomes (data not shown). This result is consistent with CEs being responsible for the pyrethroid-hydrolytic activity in microsomes since CEs react in a 1:1 stoichiometric manner with organophosphates.

3.3. *Pyrethroid hydrolysis rates and interindividual variability*

The kinetic parameters that describe the hydrolysis of *trans*-permethrin by 11 individual human liver microsomal samples were estimated to examine the variability in pyrethroid metabolism (see Table 5). While no marked differences were observed for the estimated K_m values of *trans*-permethrin among these liver samples, the maximal rates (V_{max}) for each individual sample ranged from 0.36 to 2.94 nmol/min/mg protein with a mean (±S.D.) of 1.35 ± 0.86 nmol/min/mg protein. Thus, an eight-fold difference in pyrethroid hydrolyzing activity was observed in the human liver samples. Of the CE substrates examined, the activities of the individual microsomal samples toward *p*-NPV and *p*-NPA correlated the best with the maximal rates of *trans*-permethrin hydrolysis (V_{max}) (Table 5; $r = 0.882$ and 0.752 for *p*-NPV and *p*-NPA, respectively; $p < 0.05$, $n = 11$). Thus, the significant correlation and similar range of variation (% R.S.D.) observed for both *p*-NPV and *trans*-permethrin hydrolytic rates suggest that similar human CE isozymes are responsible for hydrolyzing these substrates.

3.4. *Immunoblotting of human liver samples*

The relative levels of hCE-1 protein in each human liver sample was measured by immunoblotting to determine if the apparent variation in hydrolytic metabolism among the individual human liver samples was related to hCE-1 protein expression. Fig. 6A shows the results of immunoblotting experiments with 11 individual microsomes and the relative density of each immunoreactive band is indicated (Fig. 6C). The data demonstrate that the individual V_{max} values for *trans*-permethrin did not correlate well with hCE-1 protein levels (see Fig. 6; $r = 0.294$, $n = 11$). Thus, it is likely that other esterases in the microsomes contribute to *trans*-permethrin hydrolysis.

Table 5 – Specific activities and pyrethroid kinetic parameters of individual human liver microsomes

Code number	*para*-Nitrophenyl ester substrates[a]		*trans*-Permethrin	
	p-Nitrophenyl valerate (μmol/min/mg)	*p*-Nitrophenyl acetate (μmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
HG74	18.98 ± 0.33	3.31 ± 0.06	10.3	1.50
HH13	2.37 ± 0.05	0.76 ± 0.02	13.4	0.52
HK37	7.73 ± 0.39	3.44 ± 0.06	22.1	1.42
HG56	23.91 ± 0.91	3.26 ± 0.03	33.4	2.94
HH64	13.54 ± 0.32	2.89 ± 0.03	9.9	0.85
HH18	24.24 ± 0.86	3.51 ± 0.15	21.4	2.82
HH47	1.56 ± 0.04	1.20 ± 0.06	12.8	0.36
HG43	7.42 ± 0.44	2.17 ± 0.08	25.9	1.34
HG32	5.56 ± 0.17	1.94 ± 0.16	16.7	0.93
HK25	4.85 ± 0.23	1.80 ± 0.17	17.1	0.65
HG03	7.24 ± 0.27	3.23 ± 0.27	18.0	1.50
Average ± S.D.	10.67 ± 8.24	2.50 ± 0.97	17.9 ± 7.4	1.35 ± 0.86
% R.S.D.	77.2	38.9	41.3	63.5
Human (pooled)[b]	7.12 ± 0.31	2.21 ± 0.04	20.7 ± 3.0	1.12 ± 0.05
Rat	7.04 ± 0.62	9.33 ± 0.19	19.2 ± 3.2	1.34 ± 0.27
Mouse	10.63 ± 4.26	7.84 ± 0.17	21.0 ± 3.8	1.71 ± 0.11

[a] Values are the average (±S.D.) of three determinations.
[b] Pool of 25 individuals (male and female).

3.5. *Inhibition of carboxylesterases*

In order to determine which human isozymes are responsible for *trans*-permethrin hydrolysis in liver microsomes we have used a selective CE inhibitor. CDMB, which inhibits hCE-2 with a high degree of specificity compared to hCE-1 when using *o*-nitrophenyl actetate as substrate [24], was also shown to selectively and potently inhibit hCE-2 hydrolytic activity when both *p*-NPV and *trans*-permethrin were used as substrates (Table 6). When inhibition constants (K_i) for *p*-NPV hydrolysis are compared, hCE-2 was inhibited 90 times more potently than hCE-1 by CDMB (Table 6). Furthermore,

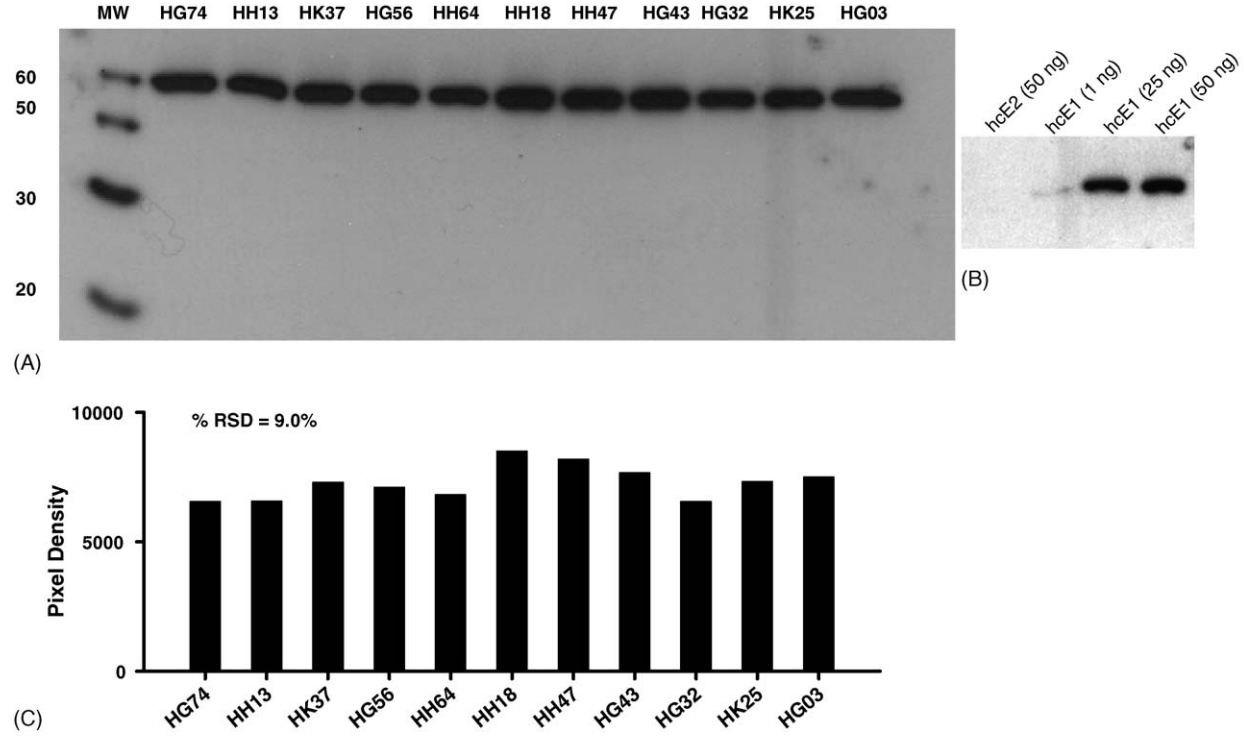

Fig. 6 – Western immunoblot of 11 individual human liver microsomal samples probed with anti-hCE-1 following SDS-PAGE. (A) Liver microsomal proteins were loaded on a 10% polyacrylamide gel and were electrophoresed. (B) Increasing amounts of purified recombinant hCE-1 protein (1–50 ng) and hCE-2 protein (50 ng) underwent SDS-PAGE and the subsequent blots were probed with anti-hCE-1. hCE-2 did not react with the anti-hCE-1 antibody. (C) Graph comparing the relative hCE-1 protein levels in each individual liver sample determined by densitometry of bands in the blot above.

Table 6 – K_i values for the inhibition of human CEs by CDMB

	$K_i \pm$ S.E. (μM)[a] (r^2)	
Enzyme	*p*-Nitrophenyl valerate	*trans*-Permethrin
hCE-1	1.13 ± 0.24 (0.9939)	6.86 ± 4.28 (0.9082)
hCE-2	0.01 ± 0.00 (0.9996)	0.19 ± 0.07 (0.9211)

[a] K_i values were determined by fitting data to an equation for a partially competitive inhibitor [24].

hCE-2 was inhibited 36 times more potently than hCE-1 when *trans*-permethrin was used as the substrate (Table 6). Thus, CDMB specifically inhibits hCE-2 activity.

When CDMB was used to inhibit metabolism by rat liver microsomes, the hydrolysis rates of bioresmethrin, *trans*-permethrin, and *cis*-permethrin were dramatically reduced ($IC_{50} \sim 2$–4 μM, see Table 7). In contrast, *p*-NPV hydrolysis by rat liver microsomes was much less sensitive to inhibition by CDMB ($IC_{50} > 100$ μM) (see Table 7). The relatively weak inhibition of *p*-NPV hydrolytic activity in rat liver microsomes may be explained by the existence of other CE isozymes in rat liver microsomes that can hydrolyze *p*-NPV, but which are insensitive to the inhibitory effects of CDMB.

Inhibition of *trans*-permethrin hydrolytic activity in human liver microsomes by CDMB yielded an IC_{50} value of 1.1 μM (Table 7). This suggested that a significant portion of the pyrethroid hydrolytic activity in microsomes was catalyzed by hCE-2. CDMB at a concentration of 1.1 μM inhibits the *trans*-permethrin hydrolytic activity of hCE-2 by 45%, whereas this concentration of inhibitor only reduced hCE-1-medated hydrolysis by ~5%. These estimates are based on parameters derived from the inhibition of pure hCE-1 and hCE-2 by CDMB (Table 6) and are consistent with hCE-1 being less sensitive than hCE-2 to the inhibitory effects of this compound [24]. Furthermore, because hCE-2 exhibits a higher affinity for *trans*-permethrin compared to hCE-1 (based on estimated K_m values, Table 2), metabolism via hCE-2 may predominate at low hepatic concentrations following environmental exposure to pyrethroids. In contrast to *trans*-permethrin, inhibition of bioresmethrin hydrolysis in human liver microsomes by CDMB was nearly 10-fold less potent (Table 7) and is consistent with the observation that hCE-2 does not hydrolyze bioresmethrin. The hydrolytic activity of human liver microsomes toward *p*-NPV was also potently inhibited by CDMB and the IC_{50} value and inhibition curve resembled that for CDMB inhibition of *trans*-permethrin hydrolysis. This was in contrast to the IC_{50} values obtained following CDMB-mediated inhibition of rat liver microsomal hydrolysis of these two substrates.

3.6. *In-gel hydrolysis assays*

Since we did not possess an antibody that could detect hCE-2 protein (see Fig. 6B), we determined relative levels of hCE-2 activity in select microsomal samples by staining native PAGE gels for esterase activity with 4-MUBA. Four individual human microsomal samples analyzed are shown in Fig. 7A. Equal amounts of microsomal protein were loaded onto native PAGE gels and both hCE-1 and hCE-2 proteins were found to be expressed in each sample; however, the intensity of staining for both hCE-1 and hCE-2 in HH13 and HH47 were markedly reduced compared to HH18. Based on the divergent relative

Table 7 – IC_{50} values for CDMB: inhibition of ester hydrolysis catalyzed by liver microsomes

Species	$IC_{50} \pm$ S.E. (μM) (r^2)			
	trans-Permethrin	*cis*-Permethrin	Bioresmethrin	*p*-Nitrophenyl valerate
Rat	4.34 ± 6.48 (0.9875)	2.38 ± 0.46 (0.9023)	2.44 ± 0.47 (0.8960)	>100 (0.9091)
Human[a]	1.08 ± 0.22 (0.9608)	–[b]	9.90 ± 0.25 (0.9477)	2.60 ± 0.36 (0.9115)

[a] Pooled human liver microsomes.
[b] Not determined.

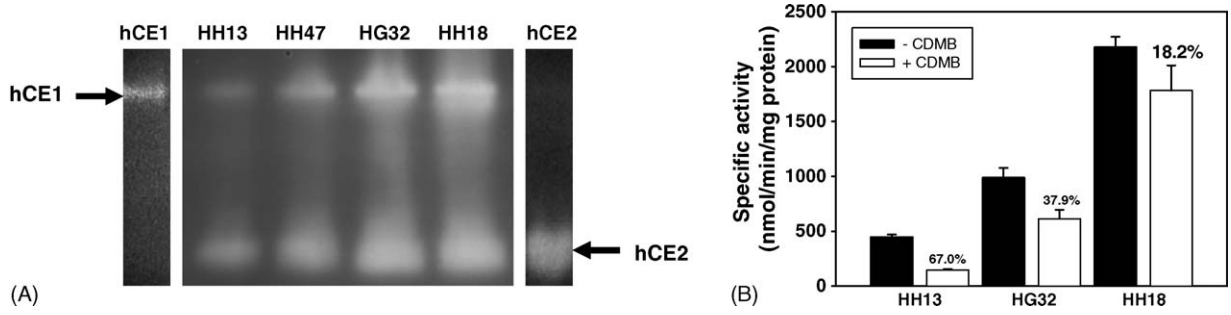

Fig. 7 – In-gel hydrolysis assay of human liver microsomal samples. (A) Four individual human liver microsomal samples (30 μg protein per lane) were fractionated on 10% native polyacrylamide gels and the esterases were detected with the substrate 4-MUBA. Each gel lane is labeled with the microsome code number. Separate gels demonstrating the migration behavior of hCE-1 or hCE-2 proteins are indicated. (B) *trans*-Permethrin specific activities of HH13, HG32, and HH18 microsomal samples in the absence or presence of CDMB (1.1 μM). The relative degree of hydrolytic inhibition (%) by CDMB is indicated over the white bar.

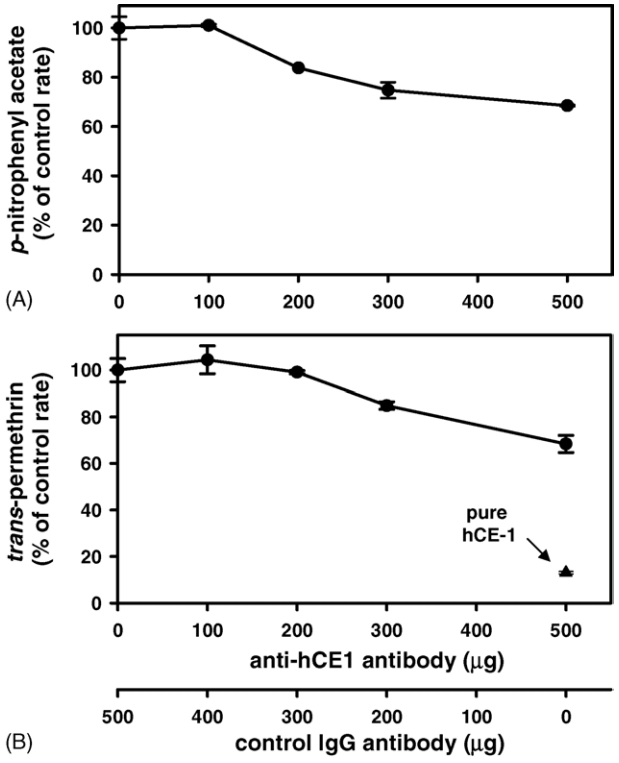

Fig. 8 – Effects of immunoprecipitation of hCE-1 with anti-hCE-1 antibody on hepatic hydrolysis activities. Solubilized pooled human liver microsomes were incubated with increasing amounts of anti-hCE-1 overnight. The total amount of IgG antibody in each reaction was 500 μg and the indicated amounts of control rabbit IgG on the abscissa was added to make up the balance. The resulting hydrolytic activity of the microsomes following immunoprecipitation toward (A) *p*-nitrophenyl acetate and (B) *trans*-permethrin was then assayed. The hydrolytic activity of pure hCE-1 was inhibited by 90% following immunoprecipitation. Each value represents the average and S.D. of two experiments. CE activity was inhibited up to a maximum of 30% for each substrate by anti-hCE-1.

inhibition potencies of *trans*-permethrin hydrolysis by CDMB in these microsomal samples (67% for HH13 and 18% for HH18, see Fig. 7B), hCE-2 appears to contribute a greater fraction of the total permethrin hydrolytic activity in the HH13 microsomes compared to the HH18 microsomes. However, HH18 has a significantly greater *trans*-permethrin hydrolytic activity than HH13 (Fig. 7B), and higher levels of both hCE-1 and hCE-2 based on the activity staining in the gel shown in Fig. 7A.

3.7. *Immunoprecipitation of hCE-1 in human liver microsomes*

Further evidence that the activity of hCE-1 did not account for the total pyrethroid hydrolytic activity in human liver microsomes was obtained from immunoprecipitation experiments. The hydrolysis of *p*-NPA and *trans*-permethrin by pooled human liver microsomes was maximally inhibited by 30% following incubation with rabbit anti-hCEl polyclonal antibody (Fig. 8A and B). Control incubations containing pure hCE-1 and anti-hCEl antibody (500 μg) resulted in a nearly 90% reduction in *trans*-permethrin hydrolytic activity (Fig. 8B), suggesting that another enzyme (e.g., hCE-2) plays a significant role in the hydrolysis of both substrates. Furthermore, these results are consistent with the CDMB inhibition data presented above, which suggested that hCE-2 or an hCE-2-like enzyme accounted for a significant fraction of the metabolism of *trans*-permethrin in human liver microsomes.

4. Discussion

Pyrethroids are increasingly being used to prevent pest infestation and for public health purposes. Hydrolysis of pyrethroids is a primary mode of their detoxication; however, little work has been done with purified esterases and pyrethroid metabolism [1,7]. The availability of recombinant human CE enzymes has enabled their substrate specificities to be characterized for a subset of pyrethroids. This study sheds light on the nature of the hydrolase enzymes in humans that metabolize pyrethroids. The two major hepatic CE isozymes (hCE-1 and hCE-2) were shown to be pyrethroid-cleaving esterases. Consistent with literature reports that described pyrethroid metabolism by rodent hepatic microsomes [31], the results obtained with the purified CEs demonstrated that type I pyrethroids are better substrates for these enzymes than type II pyrethroids, and *trans*-isomers are cleaved faster than *cis*-isomers. In some instances, e.g., bioresmethrin, the human CE enzyme that hydrolyzes the pyrethroid can be specific. Bioresmethrin hydrolysis was catalyzed efficiently by hCE-1, but not by hCE-2. Indeed, bioresmethrin may be a useful reporter probe of hCE-1 activities in human tissues because of its apparent specificity for this enzyme. The lack of sequence homology between hCE-1 and hCE-2 (only 48%) may explain some of the differences observed in the selectivity of CE-catalyzed bioresmethrin hydrolysis. However, *trans*-permethrin was hydrolyzed nearly equally efficiently by both enzymes, and both CEs likely metabolize *trans*-permethrin at low hepatic concentrations following environmental exposures. In contrast to the CEs, human acetyl-cholinesterases and butyrylcholinesterases were shown to be inefficient catalysts of *trans*-permethrin hydrolysis and likely do not contribute to pyrethroid hydrolysis. This is probably due to the fact that they are highly specific enzymes with narrow catalytic gorges compared to the CE enzymes [23]. The recombinant CEs will be useful for the study of structure-activity relationships of pyrethroid hydrolysis and will provide insight into the underlying kinetic differences found between different pyrethroids, particularly study of the stereoselective hydrolysis of the *cis*- and *trans*-isomers [38].

Comparison of hepatic microsomes from rats, mice, and humans in this study also demonstrated that the hydrolytic rates for pyrethroid chemicals were generally similar between species. Further, kinetic parameters for the rat and rabbit CEs were qualitatively similar to the human CEs when hydrolysis rates of the investigated pyrethroids were evaluated. These findings further support the use of rodent models for pyrethroid metabolism studies. Variations in the hydrolytic

rates observed in a set of individual human liver samples toward *trans*-permethrin was also found, although this variability did not appear to be related to the levels of hCE-1 protein expressed within this small population. The lack of correlation of *trans*-permethrin hydrolytic activities in individual human liver microsomes and hCE-1 protein levels suggests that other factors account for the differential permethrin-hydrolyzing activities in these human samples. This was surprising since a previous study, which had examined the levels of hCE-1 expression in 12 individual liver samples, had reported a 10-fold range in the levels of immunoreactive hCE-1 protein [35]. Furthermore, a broad range of activities toward a number of ester substrates was reported. For example, hydrolytic activities toward *p*-NPA ranged 33-fold and correlated strongly with the levels of immunoreactive hCE-1 [35]. In our study, however, the range of activities toward *p*-NPA was much narrower (approximately five-fold, Table 5) and did not correlate with the levels of hCE-1 protein (Fig. 6). The narrow range of hCE-1 protein levels among the 11 liver samples examined (% R.S.D. = 9.0%) may account for the differences between our study and that of Hosokawa et al. [35].

The use of the chemical inhibitor CDMB was shown in this study to selectively inhibit hCE-2-catalyzed *trans*-permethrin hydrolysis 36 times more potently than hCE-1. Consistent with this, CDMB also potently inhibited human liver microsomal metabolism of *trans*-permethrin, which suggests that hCE-2 has a significant role in human hepatic metabolism of this pyrethroid. In support of this, immunoprecipitation of hCE-1 from human liver microsomes only blocked up to 30% of the *trans*-permethrin hydrolyzing activity, suggesting that hCE-2 has a significant role in its metabolism. The CDMB inhibitor will be a useful tool in future studies that examine the relative contribution of hCE-2 toward pyrethroid metabolism in different human tissues. The elucidation of structures for hCE-1 and hCE-2 in complex with these benzil derivatives [36] will enable the design of more potent and selective CE inhibitors that can be utilized in pyrethroid metabolism experiments and studies that examine the metabolism of esterified xenobiotics.

In conclusion, while other unidentified esterases in humans that can hydrolyze pyrethroids may exist, this study clearly demonstrates that hCE-1 and hCE-2 are human pyrethroid-hydrolyzing carboxylesterases and that these enzymes will be useful biomarkers of susceptibility in populations that are occupationally and environmentally exposed to these xenobiotics. Furthermore, future studies that couple biochemical analyses of site-specific CE mutants with computational chemistry and/or structural approaches could provide valuable insights into the mechanisms responsible for the differential metabolism of pyrethroid stereoisomers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2005.11.020.

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