PYRETHROIDS: CYTOTOXICITY AND INDUCTION OF CYP ISOFORMS IN HUMAN HEPATOCYTES[#]

Parikshit C. Das[1], Timothy M. Streit[2], Yan Cao[1], Randy L. Rose[†], Nathan Cherrington[3], Matthew K. Ross[2], Andrew D. Wallace[1] and Ernest Hodgson[1]*

[1]*Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC,* [2]*Center for Environmental Health Sciences, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi, MS and* [3]*Department of Pharmacy, University of Arizona, Tucson, AZ, USA*

SUMMARY

Deltamethrin [(S)-α-cyano-3-phenoxybenzyl-*cis*-(1R,3R)-3(2,2-dibromovinyl)(2,2-dimethyl-cyclopropane-carboxylate] and permethrin [3-phenoxybenzyl(1RS)-*cis*,*trans*-3-(2,2-dichlorovinyl)–2,2-dimethyl-cyclopropanecarboxylate] are pyrethroid insecticides used in agriculture, public health and military deployments. Pyrethroids are known to be capable of inducing cytochrome P450 (CYP) 2B1/2B2, CYP1A1 and overall CYP content in rat liver. The objectives of this study were to evaluate the potential of deltamethrin and permethrin to cause cytotoxicity and to induce CYP isoforms in human hepatocytes. Permethrin and deltamethrin showed dose-dependent effects on adenylate kinase activity in HepG2 cells, in which 50 and 100 μM doses, respectively, induced a 3-5 fold increase in activity, and also

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[†] Deceased.

* Author for correspondence:
Dr. Ernest Hodgson
Department of Environmental and Molecular Toxicology
Mail Box 7633
North Carolina State University
Raleigh, NC 27695-7633, USA
e-mail: ernest_hodgson@ncsu.edu

induced adenylate kinase activity in primary human hepatocytes. An approximately 3-fold induction was noted at 200 μM deltamethrin and a 4-fold induction at 100 μM permethrin. Cytotoxicity was noted in HepG2 cells following 48-72 h exposure to 100 or 200 μM deltamethrin and permethrin, respectively. Dose-dependent induction of caspase-3/7 was initiated by 12.5 μM deltamethrin or by 3.125 μM permethrin. Actinomycin D, a positive control for induction of caspase 3/7, induced caspase-3/7, an effect completely abrogated by the specific inhibitor Z-DEVD-FMK. At 100 μM deltamethrin 2-3 fold induction of CYP1A1 and CYP2B6 mRNA was observed, while at the same time an ~25-fold induction of CYP3A4 was noted. Permethrin-mediated CYP induction was much less potent, 4-fold or less for CYP1A1, CYP3A4, CYP3A5, CYP2B6 and CYP2A6. It has also been shown that these pyrethroids are ligands for the pregnane X receptor (PXR).

KEY WORDS

CYP isoforms, cytotoxicity, deltamethrin, HepG2 cells, human hepatocytes, induction, permethrin, PXR/SXR

INTRODUCTION

Photostable synthetic pyrethroids were discovered in the 1970s /1/ and are widely used in agriculture, forestry, horticulture and public health, as well as in the textile industry and military deployments /2,3/. Pyrethroids are good substitutes for organochlorine, organophosphate and carbamate insecticides and are classified as safe due to their lower persistence and comparatively lower mammalian toxicity /2,4-7/.

Due to their lipophilicity pyrethroids are absorbed through the gastrointestinal and respiratory tracts and are preferentially distributed into lipid-rich body fat and nerve tissues /2/. Only a few cases of acute toxicity have been reported in occupationally exposed humans /8/. Permethrin shows estrogen and progesterone activities in human endometrial and breast cancer cells /9/.

Abbreviations: bDNA = branched DNA; CYP = cytochrome P450; PKC = protein kinase C; PXR or SXR = pregnane X receptor or steroid and xenobiotic receptor.

Metabolism studies in humans have been limited to the detection of primary pyrethroid metabolites in blood or urine /10-12/. The urinary levels of the deltamethrin metabolite 3-phenoxybenzoic acid are similar or higher in children than in adults in exposed populations /13,14/. *cis*-Permethrin is poorly metabolized by human liver fractions although the *trans*-isomer is readily metabolized, initially by both soluble and microsomal esterases, followed by alcohol and aldehyde dehydrogenases /15/. This metabolic difference between *trans*- and *cis*-pyrethroids correlates with the greater toxicity of *cis*-isomers /2/. Covalent binding of deltamethrin to hepatic microsomal proteins suggests CYP-mediated metabolism of deltamethrin /16/. Different CYP isoforms, including CYP2B1/2B2 and CYP1A1/2, are involved in deltamethrin hydroxylation /17/. Pyrethroids cause a slight induction of CYP2B1, CYP2B2, CYP1A1 and total CYP content in rat liver /17,18/. Moreover, permethrin was shown to be an inducer of CYP2B1 in isolated rat hepatocytes /19/. Recent studies have shown that permethrin, cypermethrin and fenvalerate are ligands of the pregnane X receptor (PXR) /20,21/. The aims of the present study were to evaluate the potential of deltamethrin and permethrin to cause their two most important hepatic effects, namely cytotoxicity and induction of CYP isoforms.

MATERIALS AND METHODS

Chemicals and reagents

Deltamethrin [(S)-α-cyano-3-phenoxybenzyl-*cis*-(1R,3R)-3(2,2-dibromovinyl)(2,2-dimethyl-cyclopropane-carboxylate] and permethrin [3-phenoxybenzyl(1RS)-*cis*,*trans*-3-(2,2-dichlorovinyl)–2,2-dimethyl-cyclopropanecarboxylate] were purchased from Chem Service (West Chester, PA) and Accu Standard, Inc. (New Haven, CT), respectively. Williams E culture medium and medium supplements, dexamethasone and insulin, were obtained from BioWhittaker (Walkersville, MD). EME Medium without L-glutamine and phenol red, non-essential amino acid solution, L-glutamine solution, and other cell culture related products were purchased from Mediatech Inc. (Herndon, VA). Certified fetal bovine serum, trypsin-EDTA solution and HBSS buffers were obtained from GIBCO Invitrogen Corporation (Carlsbad, CA). Tissue culture flasks, 6-well, 24-well, 48-well, and 96-well

culture plates and other tissue culture related products were purchased from Fisher Scientific Inc. (Pittsburgh, PA). FuGene 6, the *Renilla* expression vector (pRL-TK), and the dual-luciferase reporter assay system were obtained from Promega (Madison, WI). The PXR expression plasmid, pCDG1-SXR, was kindly provided by Dr. R. Evans (Salk Institute). pGL3-CYP3A4 (-7830Δ7208_364) /22/, which contains the firefly luciferase reporter gene under control by the CYP3A4 proximal and distal promoter, was generously provided by Dr. O. Burk (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Germany). The ToxiLight™ assay kit was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME). Caspase-Glo™-3/7 Assay kit was purchased from Promega Corporation (Madison, WI). Actinomycin D and Z-DEVD-FMK are products of Alexis Biochemicals supplied by AXXORA, LLC (San Diego, CA). Rifampicin, phenobarbital, 3-methylcholanthrene and all other chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Rabbit polyclonal anti-CYP2B6 antibodies and mouse monoclonal anti-CYP3A4 antibodies were purchased from BD Biosciences (Bedford, MA) and R & D Systems, Inc. (Minneapolis, MN), respectively. All chemicals, reagents and biological wastes were disposed of according to NCSU guidelines.

Human hepatocyte primary culture

Primary cultures of adult human hepatocytes were obtained from ADMET Technologies (Durham, NC). Hepatocytes were plated at 9 x 10^6 cells/plate subdivided into equal numbers in all wells of culture plates coated with type I collagen and overlaid with matrigel. The viability of cells at plating was greater than 84% as measured by the trypan blue exclusion method. Fresh hepatocytes were cultured in Williams medium E supplemented with 10^{-7} M dexamethasone, 10^{-7} M insulin, 100 U/ml penicillin G, 100 μg/ml streptomycin and 10% FBS. The cultures were maintained in a humidified incubator at 5% CO_2/95% air at 37°C for 48 h prior to the initiation of treatment.

Human hepatoma HepG2 cell line

Human hepatoma HepG2 cells were cultured in EME supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), non-essential

amino acids (0.1 mM) and L-glutamine (2 mM). Cells were maintained in a humidified atmosphere containing 5% CO_2/95% air at 37°C and were subcultured every 4-5 days. The initial culture was designated as passage number 1 and all experiments were performed using cells within the first 10 passages to minimize inter-experimental variability and to maximize intra-experimental data reproducibility.

Cell culture-based luciferase reporter gene assay

The HepG2 cells were transfected with three plasmids, a PXR expression vector, a plasmid containing the luciferase reporter gene, and a plasmid containing the *Renilla* luciferase gene, the latter as a transfection efficiency control. Transfection of HepG2 cells was performed based on a recently published method describing the dual-luciferase assay in the HepG2 cell line /23/. Briefly, HepG2 cells were seeded into a 12-well plate at a density of ~500,000 cells/well. One day post-seeding, the cells (~50-80% confluent) were co-transfected with pGL3-CYP3A4, pCDG1-SXR, and pRL-TK plasmids for 24 h. The transfection reagent FuGene 6 and plasmid DNA were mixed and incubated together according to the manufacturer's protocol. Transfection solutions per well consisted of the pGL3-CYP3A4, pCDG1-SXR, and pRL-TK plasmids in the amounts of 360, 90, and 10 ng, respectively, along with 540 ng sonicated salmon sperm DNA to reach a total DNA amount of 1 μg. In select cases, HepG2 cells were mock transfected with PXR expression vector.

Treatment of human primary hepatocytes and HepG2 cells

Human hepatoma HepG2 cells were plated at 2 x 10^4 cells/well in 96-well plates and treated with vehicle (DMSO), phenobarbital (100 μM) and actinomycin D (1 μM) (positive controls), and increasing concentrations of permethrin (25-200 μM) or deltamethrin (12.5-200 μM) for 24, 48 and 72 h. For the caspase-3/7 assay, HepG2 cells were treated with permethrin or deltamethrin (3-200 μM) for the same duration.

Fresh human hepatocytes seeded at 1.875 x 10^5 cells/well in 48-well plates were used for ToxiLight and caspase-3/7 assays following exposure to vehicle controls, positive controls such as phenobarbital (100 μM) and actinomycin D (1 μM), inhibitor Z-DEVD-FMK (0.05 μM), permethrin (3-200 μM) or deltamethrin (12.5-200 μM) for 24,

48, and 72 h. Again, fresh human hepatocytes were seeded at 1.5 x 10^6 cells/well in 6-well plates and at 3.75 x 10^5 cells/well in 24-well plates. Primary hepatocytes were exposed for 72 h to vehicle, inducing agents such as rifampicin (10 μM) or 3-methyl cholanthrene (10 μM), and increasing concentrations of permethrin (5-100 μM) or deltamethrin (10-100 μM) for the branched DNA (bDNA) and Western blot analyses, and for the CYP1A1 and CYP3A4 enzyme activity assays.

Transfected HepG2 cells were treated for 24 h with fresh medium containing 10 μM of the following compounds: rifampicin, deltamethrin, permethrin or cypermethrin in the first set of experiments; and 10 μM of the following compounds: rifampicin, bioresmethrin, *trans*-permethrin, *cis/trans* (50/50) permethrin, *cis*-permethrin, cypermethrin, cyhalothrin, or esfenvalerate in the second set of experiments. The amount of vehicle DMSO in the medium was always 0.1% (v/v).

Sample preparation

Culture supernatant was collected from 96-well and 48-well plates for use in the ToxiLight assay, while a mixture of both culture medium and cells was used for the caspase-3/7 assay. Cells in 96-well plates were harvested as suspensions using 2-5 μl trypsin-EDTA for the trypan blue exclusion assay. Cells were harvested using a cell scraper and pooled in Eppendorf tubes for extraction of protein and total RNA, respectively. Following centrifugation at 5,000 *g* for 3 min the supernatant was discarded. The cells were re-suspended in 75 μl chilled CYP storage buffer (0.1 M potassium phosphate buffer with 0.1 mM EDTA, pH 7.5) and sonicated twice for 30 s. S9 fraction was obtained by centrifuging the homogenate at 9,000 *g* for 15 minutes. Following treatment of transfected HepG2 cells, the culture medium was removed and the cells were washed twice with PBS prior to being lysed with 250 μl of passive lysis buffer (Promega). Lysates were stored at -20°C until the luciferase activity assays were performed.

Cell viability and/or cytotoxicity assay

Cell viability was assessed using cell suspensions in isotonic culture medium by the trypan blue exclusion assay. Specifically, 100 μl of 0.4% trypan blue in PBS, pH 7.4, was added to 900 μl of cell suspension. Ten μl of this mixture was placed on the hemocytometer

and more than 100 cells per field were examined to determine the percentage of dead versus viable cells.

Adenylate kinase assay

ToxiLight™ bioassay is a non-destructive luciferase-based bioluminescence cytotoxicity assay used to measure toxicity in mammalian primary cells and cell lines in culture. This assay quantitatively measures the release of adenylate kinase into the culture medium. The emitted luminescence intensity expressed as RLU (relative luminescence unit) is linearly related to the adenylate kinase activity released into the medium. The assay was performed according to the manufacturer's protocol (Lonza Rockland, Inc., Rockland, ME). Measurements at different time points are cumulative from the zero time point.

Caspase-3/7 assay

Caspase-Glo™-3/7 assay is a homogeneous luciferase based bioluminescence assay that quantitatively measures caspase-3/7 activities using a luminometer. Intensity of the luminescence produced by luciferase is proportional to the amount of caspase-3/7 activity present in the sample mixture. The assay was performed according to the manufacturer's protocol (Tech. Bull. No.323, Promega Corporation, Madison, WI).

Branched DNA (bDNA) assay

The bDNA assay is a measure of mRNA induction /24,25/. Oligonucleotide probe sets specific for human CYP1A1, CYP1A2, CYP3A4, CYP3A5, CYP2B6, CYP2D6, CYP2A6 and glyceraldehyde phosphate dehydrogenase were a generous gift from Xenotech LLC (Olathe, Kansas). Reagents required for RNA analysis (i.e., lysis buffer, amplifier/label probe buffer and substrate solution) were supplied in the QuantiGene Discover kit (Genospectra, Fremont, CA). Expression levels were analyzed as described by Hartley and Klaassen /24/. Briefly, specific oligonucleotide probe sets were diluted in lysis buffer. Cell lysate was vortexed and 20 µl was added to each well of a 96-well plate containing capture hybridization buffer (0.05 M HEPES sodium salt, 0.05 M HEPES free acid, 0.037 M lithium lauryl sulfate, 0.5 % [v/v] Micr-O-protect, 8 mM EDTA, 0.3 % [w/v] nucleic acid

blocking agent) and 50 µl diluted probe set (50, 100, and 200 fmol/µl for capture, blocker, and label probes, respectively). RNA was allowed to hybridize to each probe set containing all probes for a given transcript (blocker probes, capture probes, and label probes) overnight at 53°C. Subsequent hybridization and post-hybridization wash steps were carried out according to the manufacturer's instructions, and luminescence was measured with the Quantiplex 320 bDNA Luminometer (Bayer Diagnostics) interfaced with Quantiplex Data Management Software Version 5.02 (Bayer Diagnostics) for analysis of luminescence from 96-well plates. CYP expression data was normalized with respect to glyceraldehyde phosphate dehydrogenase levels and expressed as induction relative to controls.

Gel electrophoresis, immunodetection and quantification

7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve microsomal proteins based on the method of Laemmli /26/. Proteins from the gel were electrophoretically transferred to a nitrocellulose membrane at constant voltage (100 V) for 1 hour, and stained with Ponceau S for verification of transfer. The blots were blocked in 1% nonfat dry milk for 12 h and incubated in primary monoclonal anti-human CYP3A4 antibody and secondary anti-mouse IgG antibody conjugated to alkaline phosphatase. Specific protein bands were visualized in BCIP/NBT. The intensities of the bands on a film were measured using densitometry and data expressed on a quantitative scale.

Dual-luciferase assay

To measure the firefly luciferase activity of the lysates, 30 µl of luciferase assay reagent II (Promega) was mixed with 5 µl of lysate and luminescence was measured using a TD-20/20 luminometer (Turner Designs). The firefly luciferase reaction was quenched by the addition of 30 µl of Stop and Glo reagent (Promega) followed by detection of luminescence derived from *Renilla* luciferase activity. Firefly luciferase activity was normalized to the *Renilla* luciferase activity in each sample. Two and three independent transfections were performed with triplicate treatments for each compound for the first and second set of experiments (n = 6 and n = 9), respectively. Induction relative to the DMSO control was averaged for each compound.

Statistical analysis

Data were summarized and expressed as means ± SE using Microsoft Excel spreadsheet and Sigma Plot graphics program (Chicago, IL, USA). The significant differences between control and treated data sets were determined using Student's t-test.

RESULTS

Effect of deltamethrin and permethrin on adenylate kinase activity in HepG2 cells and human hepatocytes

The induction and subsequent release of adenylate kinase into the culture medium is an indicator of cytotoxic damage to cells. Permethrin and deltamethrin had a dose-dependent effect on the release of adenylate kinase activity from cultured human HepG2 cells; 50 and 100 μM doses caused the highest release, ~3- and 5-fold, respectively. At the highest (200 μM) dose of either pyrethroid, adenylate kinase release was somewhat lower (Fig. 1). Permethrin and deltamethrin also induced adenylate kinase in human primary hepatocytes in a time- and dose-dependent manner, an ~3-fold induction being noted at the highest (200 μM) dose of deltamethrin, and an ~4-fold induction at the 100 μM dose. 200 μM permethrin caused reduced activity (Fig. 2).

Toxicity of deltamethrin and permethrin in HepG2 cells

The trypan blue assay was used to correlate adenylate kinase activity with cell cytotoxicity caused by permethrin and deltamethrin. The data showed 15-25% cytotoxicity after 48 -72 h exposure to 100 or 200 μM deltamethrin, and 15-20% cell death at 100 or 200 μM permethrin in HepG2 cells (Fig. 3).

Effect of deltamethrin and permethrin on caspase-3/7 activity in HepG2 cells and human hepatocytes

Induction of activated caspase-3/7 is an indicator of cellular apoptosis. In order to characterize whether pyrethroid-mediated cell death was triggered through this known apoptotic pathway, cultured human HepG2 cells were exposed to increasing doses of deltamethrin and permethrin (0-200 μM) for 24, 48 and 72 h, and caspase-3/7

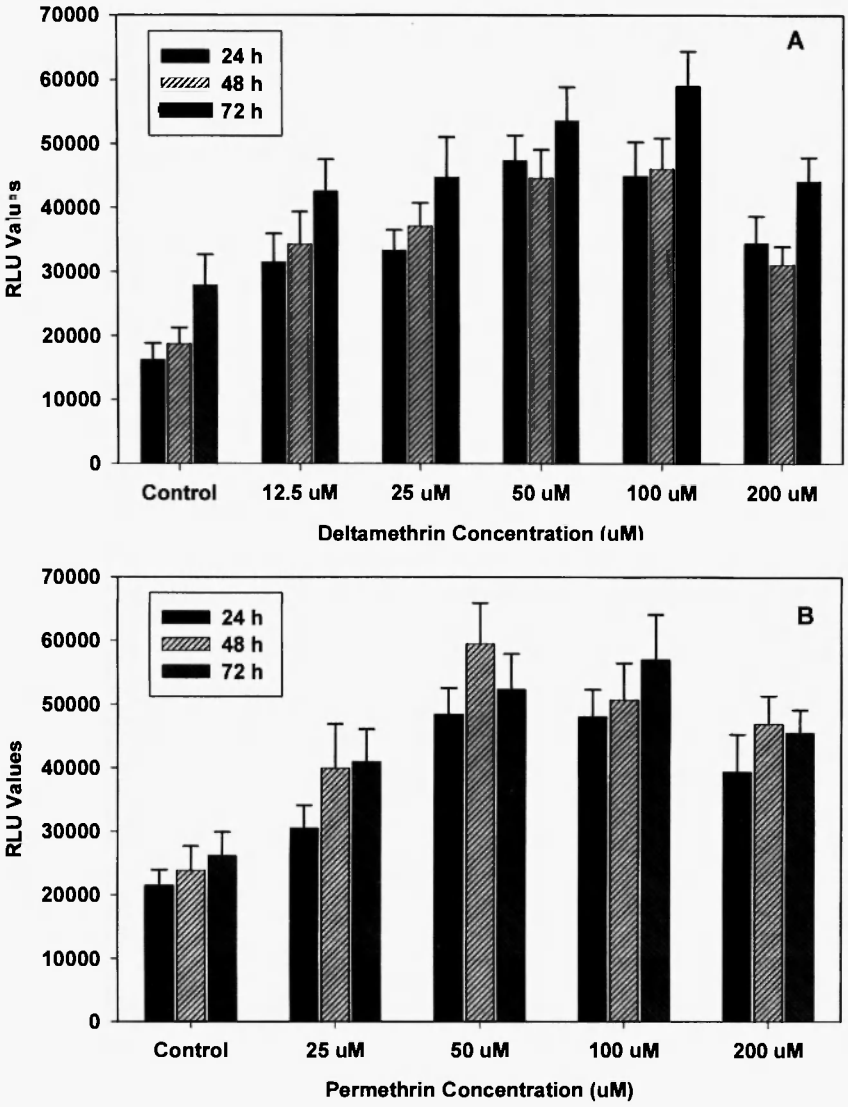


Fig. 1: Dose- and time-dependent effect of deltamethrin (**A**) and permethrin (**B**) on the adenylate kinase activity of HepG2 cells. Cultured HepG2 cells were exposed to increasing concentrations of deltamethrin (0, 12.5, 25, 50, 100 and 200 μM) or permethrin (0, 25, 50, 100 and 200 μM) for 24, 48 and 72 h and adenylate kinase activity was measured using the ToxiLight assay kit. Each bar represents the RLU value mean of nine determinations from three independent experiments in HepG2 cells. The error bars represent the standard error of the mean.

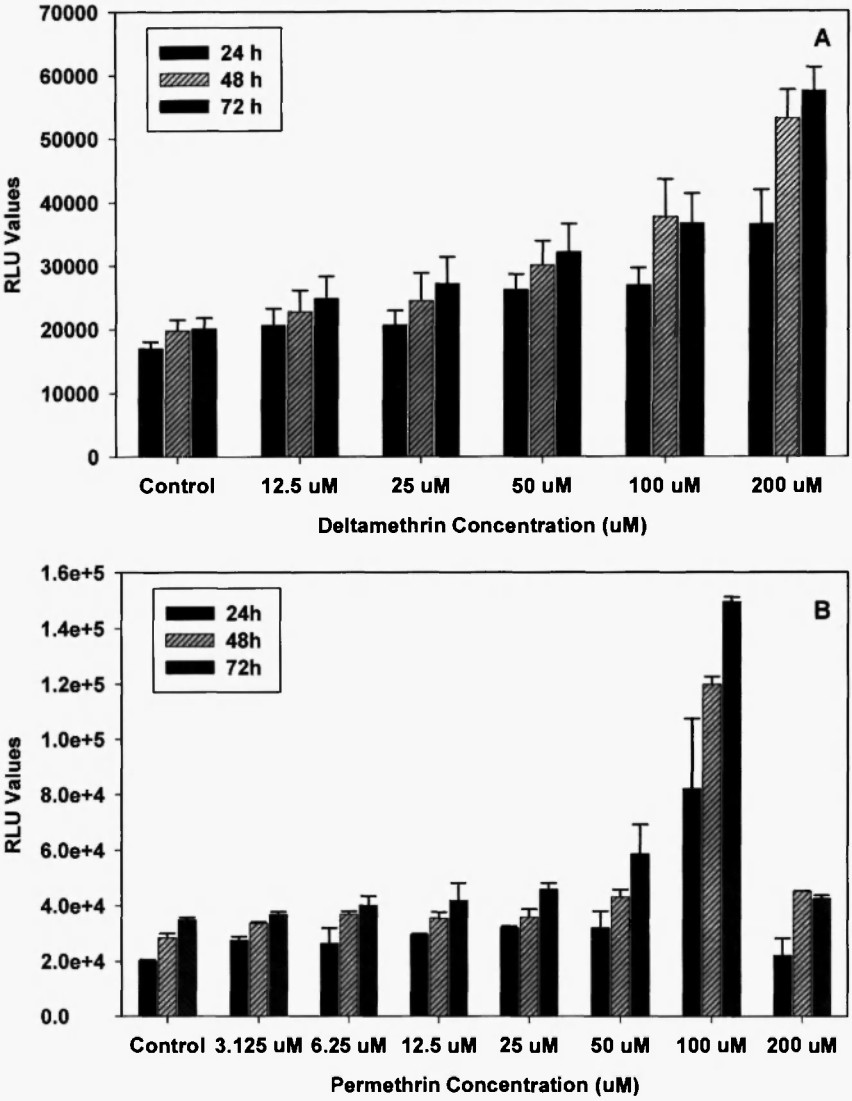


Fig. 2: Dose- and time-dependent effect of deltamethrin (**A**) or permethrin (**B**) on the adenylate kinase activity of primary cultures of human hepatic cells. Cultured hepatocytes were exposed to increasing concentrations of deltamethrin (0, 12.5, 25, 50, 100 and 200 μM) or permethrin (0, 3.125, 6.25, 25, 50, 100 and 200 μM) for 24, 48 and 72 h and adenylate kinase activity was measured using the ToxiLight assay kit. Each bar represents the RLU value mean of 6-8 determinations from two individuals. The error bars represent the standard error of the mean.

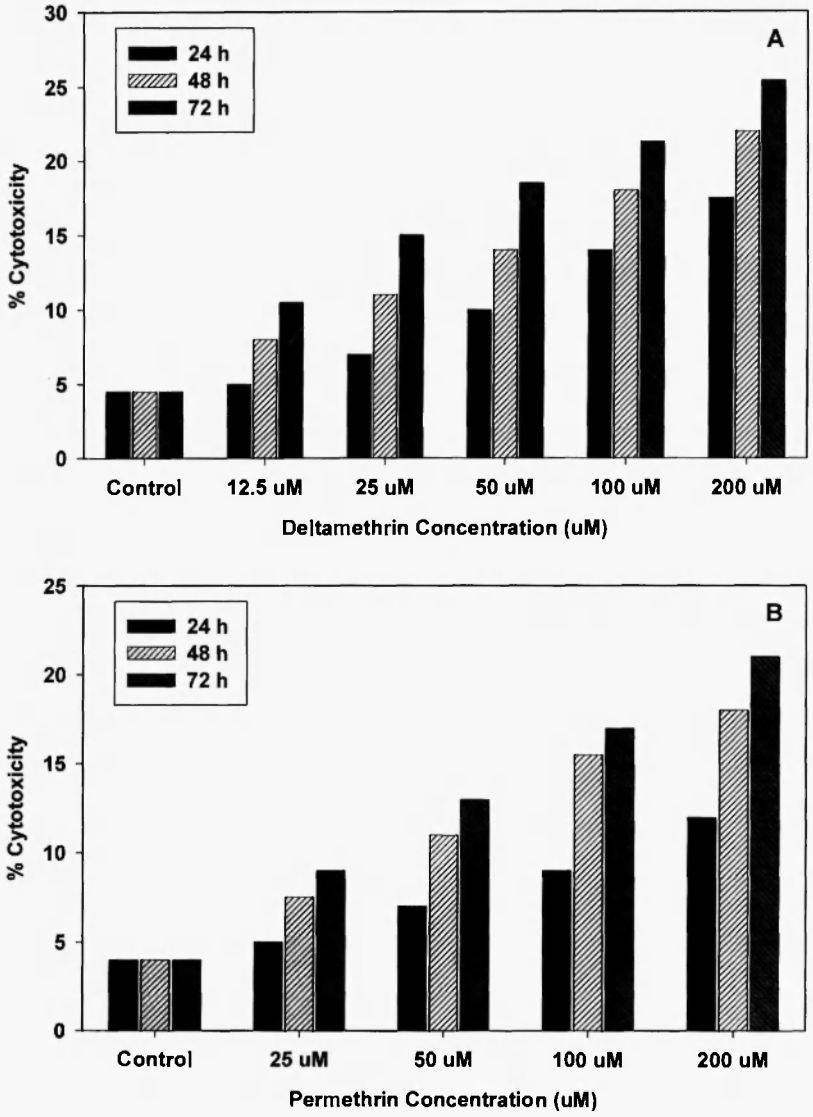


Fig. 3: Dose- and time-dependent effect of deltamethrin (**A**) or permethrin (**B**) on human hepatoma HepG2 cell viability. Cultured HepG2 cells were exposed to increasing concentrations of deltamethrin (0, 12.5, 25, 50, 100 and 200 μM) and permethrin (0, 25, 50, 100 and 200 μM) for 24, 48 and 72 h and cell viability was assessed by the trypan blue assay and expressed as % cytotoxicity. Each bar represents the value derived from counting 150-200 cells in each treated sample.

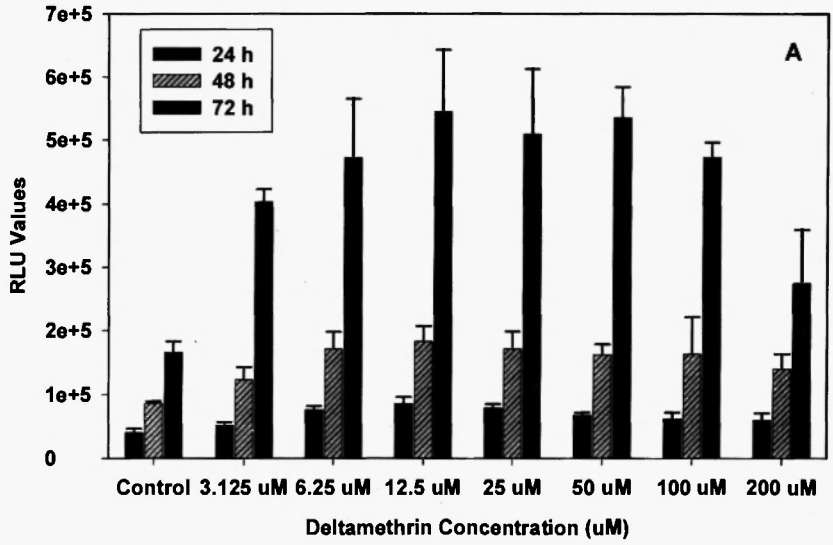
activity was measured. A dose-dependent increase in the induction of activated caspase-3/7 was noted from the lowest dose of 3.125 μM to a maximum at 12.5-50 μM, approaching a plateau or decreasing at 200 μM after 72 h. Approximately 2-3-fold activation was noted for both chemicals at 72 h (Fig. 4). Primary cultures of human hepatocytes were also exposed to increasing doses of deltamethrin and permethrin for up to 72 h. A single dose of a known inducer such as actinomycin D (1 μM) and the well known caspase-3/7 inhibitor Z-DEVD-FMK (0.05 μM) served as positive controls. Deltamethrin showed ~2-fold induction at 100 and 200 μM, while permethrin showed 1.5-2 fold induction in caspase-3/7 at 25-100 μM decreasing at 200 μM from 48 to 72 h in primary hepatocytes (Fig. 5). Actinomycin D induced caspase-3/7 about 5-7 fold whereas Z-DEVD-FMK abrogated the pyrethroid-induced caspase-3/7 activity (data not shown).

Effect of deltamethrin and permethrin on CYP isoform mRNA transcripts in human hepatocytes

In order to determine effects on CYP gene expression, primary human hepatocytes were exposed to 100 μM of deltamethrin or permethrin for 72 h. The data showed induction of mRNA expression of CYP1A1, CYP3A4 and CYP2B6 isoforms. 2-3 fold induction of CYP1A1 and CYP2B6 was noted while ~25-fold induction of CYP 3A4 was noted with deltamethrin (Fig. 6A). However, substantial variability among individuals, as yet unexplained, was noted in the induction of CYP isoform mRNAs. Permethrin-mediated CYP isoform induction was less potent. Up to 4-fold induction was noted for CYP1A1, CYP3A4, CYP3A5, CYP2B6 and CYP2A6 mRNA (Fig. 6B). Permethrin also showed substantial variability in CYP induction.

Effect of deltamethrin and permethrin on CYP3A4 protein in human hepatocytes

In order to verify the potential of deltamethrin and permethrin to induce CYP3A4-specific protein expression, Western blot analysis was performed and CYP3A4-specific protein was measured semi-quantitatively in primary human hepatocytes following treatment with deltamethrin (0-100 μM) or permethrin (0-100 μM) for 72 h. Even though induction was shown to be dose-dependent, inter-individual



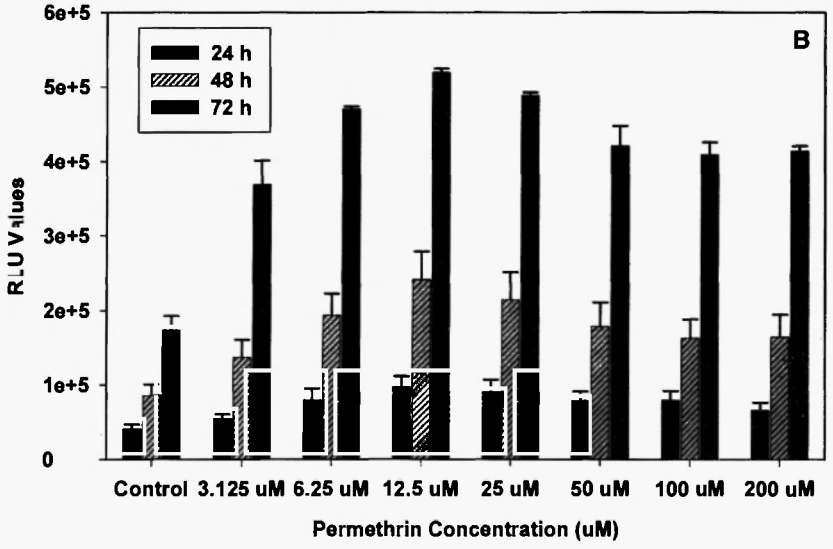


Fig. 4: Dose- and time-dependent effects of deltamethrin (**A**) or permethrin (**B**) on caspase-3/7 activity in human hepatoma HepG2 cells. Cultured HepG2 cells were exposed to various concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM) of deltamethrin or permethrin along with actinomycin D (1 μM), an inducer of apoptosis, and the caspase-3/7 specific inhibitor Z-DEVD-FMK (0.05 μM) for 24, 48 and 72 h, and caspase-3/7 activity was quantitatively measured by the Caspase-Glo®-3/7 assay kit. Each bar represents the mean RLU value of two independent experiments, 3-4 well determinations per group per experiment. The error bars represent the standard error of the mean.

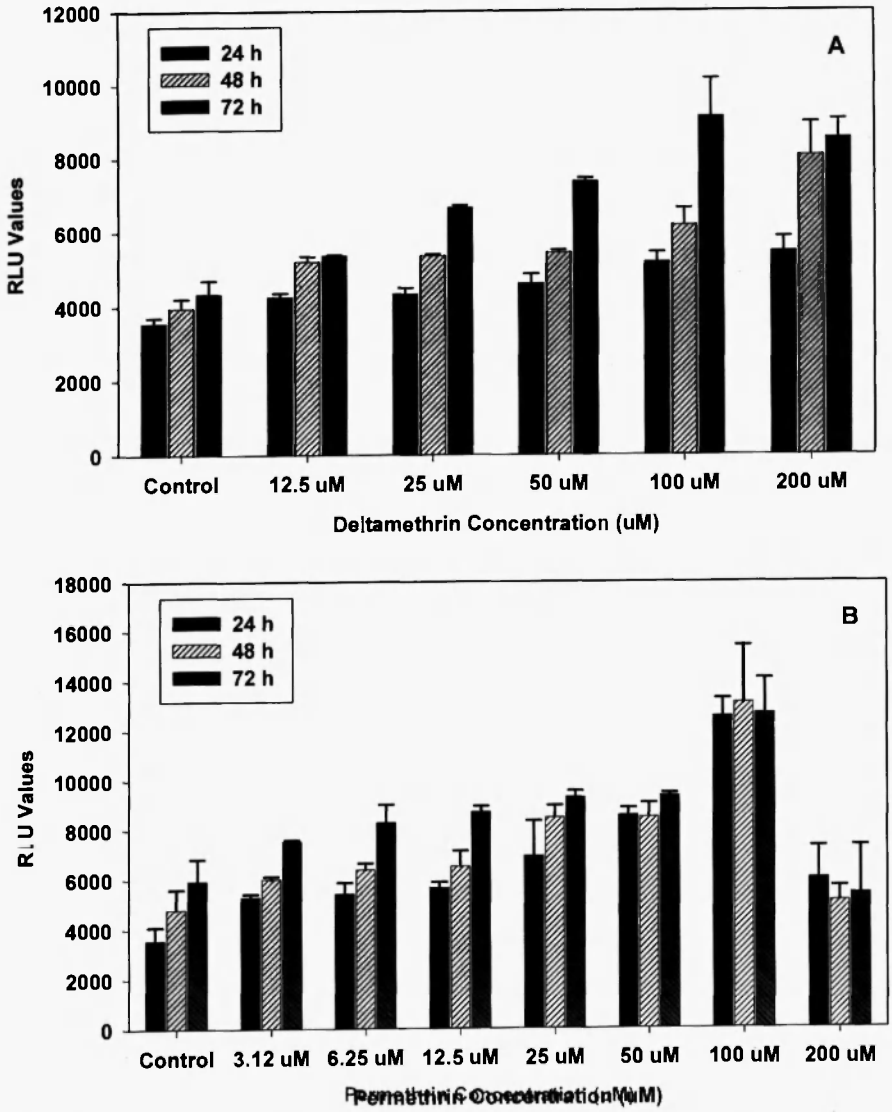


Fig. 5: Dose- and time-dependent effect of deltamethrin (**A**) or permethrin (**B**) on caspase-3/7 activity in human primary hepatocytes. Primary cultures of hepatocytes were exposed to various concentrations of deltamethrin (0, 12.5, 25, 50, 100 and 200 μM) or permethrin (0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μM) with actinomycin D (1 μM), an inducer of apoptosis, or the caspase-3/7 specific inhibitor Z-DEVD-FMK (0.05 μM) for 24, 48 and 72 h, and caspase-3/7 activity was quantitatively measured using the Caspase-Glo®-3/7 assay kit. Each bar represents the mean RLU value from six determinations (two individuals, three wells each). The error bars represent the standard error of the mean.

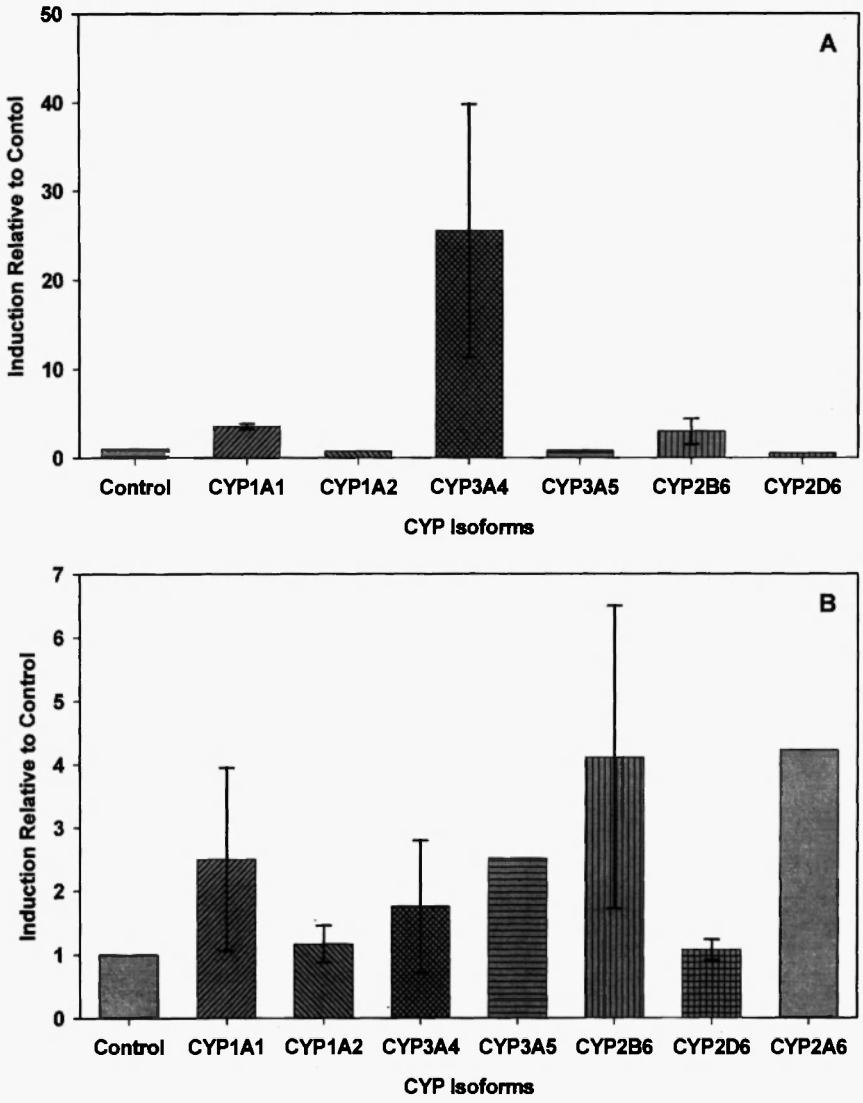


Fig. 6: Effect of deltamethrin (**A**) or permethrin (**B**) on the expression of mRNA transcripts of different CYP isoforms in human hepatocytes. Freshly prepared human hepatocytes from different individuals were exposed to 100 μM deltamethrin or permethrin, respectively, for 72 h and mRNA transcripts were measured by the bDNA assay. Each bar represents the mean mRNA value of each isoform from six determinations (two individuals, three wells each). Appropriate positive controls were included in the experiment. The error bars represent the standard error of the mean; the absence of an error bar indicates a single determination.

variability in induction reduced the significance of the CYP3A4 protein expression findings (Fig. 7).

Effect of deltamethrin and permethrin on nuclear receptor PXR in transiently transfected HepG2 cells

To determine whether the induction of CYP mRNA following deltamethrin or permethrin treatments is mediated via a PXR mechanism, a dual-luciferase assay was performed using HepG2 cells. Co-transfected cells were treated with 10 μM deltamethrin, 10 μM permethrin or 10 μM rifampicin along with other pyrethroids and sham controls with DMSO (final concentration, 0.1% v/v) for 24 h. Induction profiles for rifampicin, deltamethrin, cypermethrin and DMSO treated controls, along with the appropriate PXR-mock transfection treatments, are shown in Figure 8A. Similarly, induction profiles for another set of pyrethroids (rifampicin, bioresmethrin, *trans*-, *cis*/*trans*- and *cis*-permethrin, cypermethrin, deltamethrin, cyhalothrin, esfenvalerate and DMSO) are shown in Figure 8B. As expected, the rifampicin positive control exhibited a PXR-dependent induction of luciferase following activation of the CYP3A4 promoter. PXR dependence was verified by the lack of rifampicin-mediated induction when the PXR expression vector was absent. Likewise, the pyrethroids exhibited PXR-dependent induction. Except for bioresmethrin, the various pyrethroids tested were shown to induce CYP3A4 promoter activity by several-fold. This study demonstrates that deltamethrin and permethrin can activate CYP3A4 promoter activity by acting as potent PXR ligands. Specifically, deltamethrin treated cells exhibited an ~5-fold increase and permethrin treated cells showed ~4-10 fold increase in luciferase activity as compared to the vehicle treated control.

DISCUSSION

Because of the widespread use of pyrethroids this study was designed to examine the potential toxicity and metabolic impact of pyrethroids on human liver cells. Deltamethrin, an α-cyano type II pyrethroid, induced adenylate kinase activity in both human hepatoma HepG2 cells and primary hepatocytes. Activated adenylate kinase can trigger ATP synthesis, which in turn may enhance the hydrolysis of

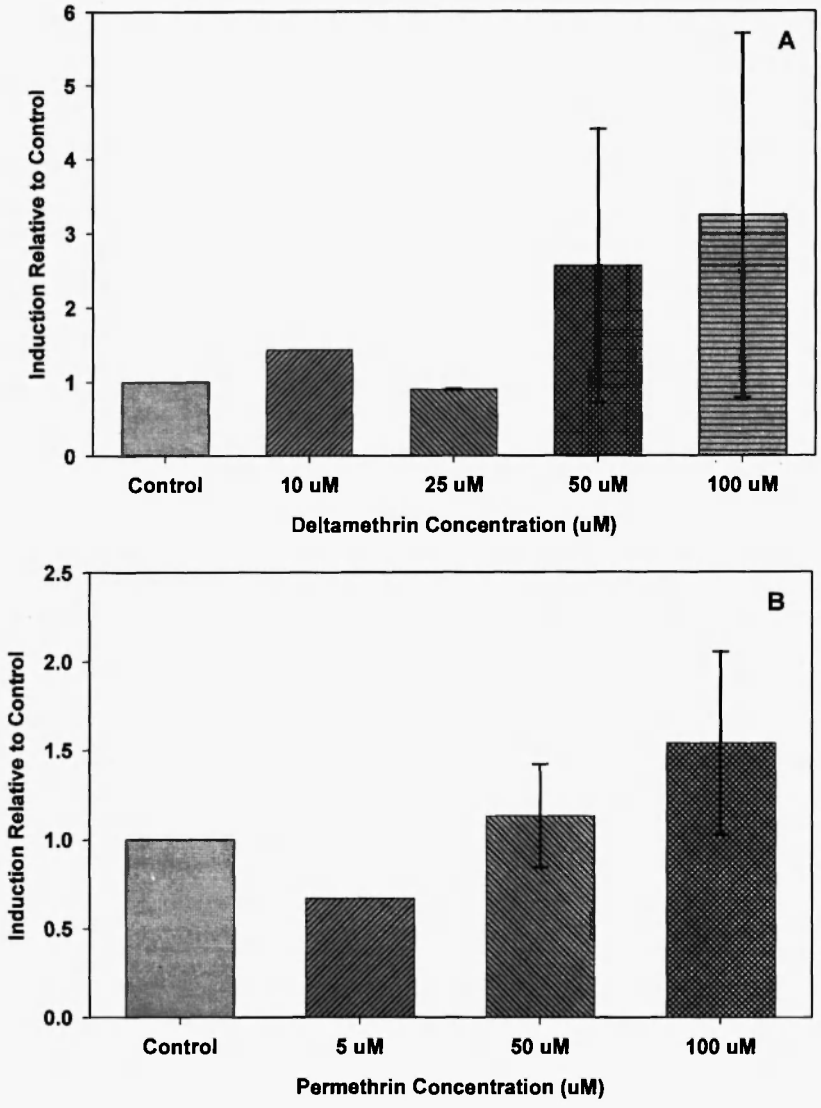


Fig. 7: Dose-response effect of deltamethrin (**A**) or permethrin (**B**) on CYP3A4 protein in fresh human hepatocytes. Freshly prepared donor human hepatocytes from different individuals were exposed to increasing concentrations of deltamethrin (0, 10, 25 and 100 μM) or permethrin (0, 5, 50 and 100 μM) for 72 h and CYP3A4 protein was determined by Western blot analysis from hepatocytes from 2-4 individuals donors. The graph represents the mean of semi-quantitative densitometric data at different doses. The error bars represent the standard error of the mean.

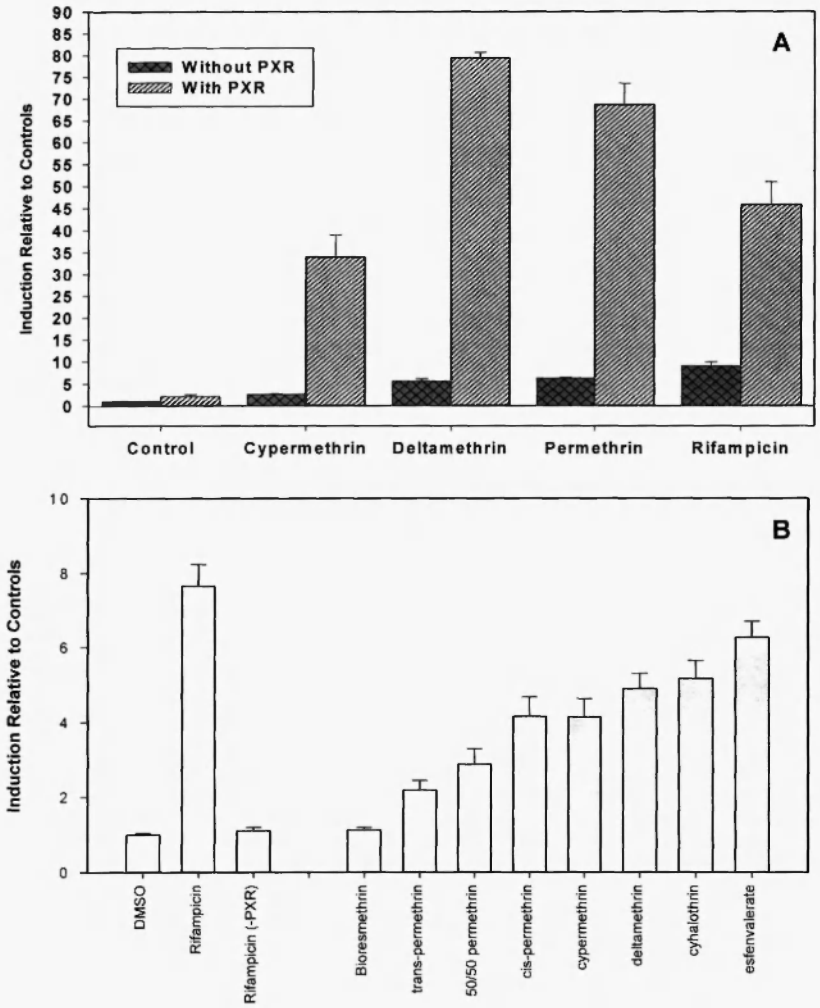


Fig. 8: Effect of deltamethrin, permethrin, and a few other pyrethroids on the induction of CYP3A4 promoter luciferase activity in dual-transfection HepG2 cells. Dual-transfection HepG2 cells were treated with a variety of pyrethroids including deltamethrin (50 μM), permethrin (50 μM), and cypermethrin (50 μM) (**A**), as well as bioresmethrin (10 μM), trans-, 50/50 cis/trans-, and cis-permethrin (10 μM each), cypermethrin (10 μM), deltamethrin (10 μM), cyhalothrin (10 μM), and esfenvalerate (10 μM) (**B**) for 24 h, and luciferase activity was determined by dual-luciferase assay from five independent experiments (two and three experiments, respectively, for each set of data). The graph represents the mean ± SE (where n = 6 and n = 9 for A and B, respectively) of relative induction of luciferase activity at different doses.

phosphoinositide to generate inositol phosphates and diacylglycerol that trigger the mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC). Pyrethroids have been reported to activate the PKC/phosphoinositol pathway in rat brain tissue /27/. Deltamethrin or permethrin may thus trigger ATP formation through a cascade of second messenger signaling mechanisms to activate PKC, which are involved in the cellular regulation of programmed cell death or apoptosis /28/.

Caspases are also involved in the initiation and transduction of apoptotic signals /29,30/. Pyrethroids induce oxidative stress and alter antioxidant systems in rat tissues /31-33/. Formation of oxidative metabolites causes release of cytochrome *c* and other polypeptides that bind to the apoptotic protease activating factor-1, causing ATP- or dATP-dependent binding to the prodomain of procaspase-9 /34/. The proteolytic activity of procaspase-9 initiates a protease cascade /35,36/, resulting in activation of caspases-3/7. Short- and long-term exposure to deltamethrin stimulates lipid peroxidation in mice /37/, and induces neurodegeneration and apoptotic cell death in rats /38/. In the present study exposure of human hepatoma HepG2 and primary hepatocytes to deltamethrin (up to 100 μM) significantly induced caspase-3/7 activity, while permethrin induced activity at all doses (3.12 to 200 μM). Pyrethroid-mediated caspase-3/7 induction may occur via mitochondrial oxidative stress (see above).

Pyrethroids such as deltamethrin appear to impact kinase- and phosphatase-mediated signal transduction pathways. For example, in astrocytes deltamethrin can inhibit the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (protein phosphatase 2B [PP2B]). /39/. Deltamethrin-mediated enhancement of ATP-induced intracellular Ca^{2+} release via the inositol-1,4,5-phosphate receptor (IP_3R) is regulated by calcineurin-mediated dephosphorylation in COS-7 cells /40-42/. In endothelial cells, deltamethrin acts as an inhibitor of PP2B /43-46/. Pyrethroids have estrogenic and antiprogestogenic effects in human tumor cells /9,47/, and pyrethroid metabolites are capable of interacting with the estrogen receptor /48/.

Signaling pathways involving PKC repress PXR activity by altering the PXR–co-factor protein complex in mouse hepatocytes /49/. Recent studies have demonstrated that various pesticides are capable of activating hPXR, although permethrin has a lower affinity for hPXR than the positive control rifampicin /21/. Activated hPXR

forms a heterodimer with the retinoid X receptor (RXR), this complex interacting with a xenobiotic-responsive element found in the promoter regions of genes that regulate multiple drug-metabolizing genes /50/, including CYP3A4 and CYP2B6 /51-54/. Permethrin elicits the most pronounced induction of CYP2B1 and CYP3A1 mRNA and these effects occurred at the transcriptional level /19/. In our study, pyrethroids elicited mixed effects on CYP-encoded mRNA expression. In general, deltamethrin and permethrin exhibited low inducibility of CYP-specific mRNA and the effects were extremely variable for different CYPs. The induction of CYP-isoforms CYP1A, CYP3A4 and CYP2B6 by deltamethrin and permethrin in human hepatocytes may be due to variable effects on transcription signaling mechanisms. Much of the variation is presumably due to inter-individual variation in transcription factor levels between hepatocyte donors /55/. Further research is necessary to resolve this question.

Similar to previous studies /20,51,54/, rifampicin exhibited a PXR dependent induction of luciferase activity by activation of the CYP3A4 promoter. This study further demonstrated that deltamethrin and permethrin are potent PXR agonists. Specifically, deltamethrin treated cells exhibited 5-15 fold and permethrin 4-10 fold increases in CYP3A4 promoter activity compared to vehicle treated cells. We have demonstrated for the first time that deltamethrin and permethrin are ligands of PXR. *trans*-Permethrin showed less induction of luciferase activity compared to deltamethrin and *cis*-permethrin, corroborating recent findings /21/. Thus, deltamethrin and permethrin acting as PXR ligands are capable of triggering CYP-specific transcription and translation. CYP3A4 is the most abundant and important CYP isoform in human liver, has broad substrate specificity, and metabolizes ~60% of clinical drugs and other chemicals /56,57/. CYP2B6 is also important in pesticide metabolism /58/, thus changes in functional CYP3A4 and CYP2B6 expression by deltamethrin and permethrin may have implications for human health risk assessment.

It is important to note that although pyrethroids have been in use as alternatives to organochlorine, organophosphate and carbamate insecticides and are believed to be only mildly toxic to mammals, they are not free from adverse effects /59/. Pyrethroids are transmitted through breast milk /60/ and possess anti-androgenic and anti-estrogenic activity /61,62/. Pyrethroids moderately induce CYP1B1/2B2 and CYP1A1 which are directly involved in xenobiotic

metabolism, including the bioactivation of carcinogens in a variety of cell types. As discussed above, pyrethroid-mediated induction of mitochondrial oxidative stress, apoptotic cell death and the potential to affect hormonal and immune suppression have been shown in a number of *in vitro* and *in vivo* mammalian studies. Deltamethrin and permethrin can act as ligands for PXR and show moderate effects on the major metabolic enzymes, and they induce apoptotic cytotoxicity in human hepatocytes. Overall, our findings further emphasize that pyrethroid insecticides may impact human health and require further analysis and assessment.

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