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Induction of cytochrome P450 2B1 by pyrethroids in primary rat hepatocyte cultures

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Abstract

Numerous xenobiotics are capable of inducing their own metabolism and by enzyme induction can also lead to enhanced biotransformation of other xenobiotics. In this project, we examined the influence of pyrethroids (permethrin, cypermethrin, and fenvalerate) on the expression and activity of the phenobarbital (PB)-inducible cytochrome P450 2B1 isoform (CYP2B1) in primary rat hepatocyte cultures. Incubation of hepatocyte cultures with pyrethroids resulted in a marked CYP2B1 induction. Among the tested pyrethroids, permethrin elicited the most pronounced induction of CYP2B1 mRNA, which exceeded maximal induction achieved by PB at concentrations approximately 10-fold higher. Furthermore, permethrin induced CYP3A1 mRNA expression, while the expression of the CYP1A1 isoform, which *in vivo* is not responsive to PB treatment, was not significantly affected by pyrethroids. Permethrin-dependent enhancement of CYP2B1 and CYP3A1 mRNA expression was repressed by the hepatotrophic cytokine epidermal growth factor, which is known to also inhibit PB-dependent induction of CYP2B1. Several metabolites of permethrin formed by hepatocytes (3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid, 3-phenoxybenzyl alcohol, and 3-phenoxybenzoic acid) were ineffective in inducing CYP2B1 mRNA. Furthermore, permethrin stimulated the expression of the luciferase reporter gene under control of the *CYP2B1* promoter (comprising the PB-responsive enhancer module) in transiently transfected primary hepatocyte cultures. Thus, permethrin-stimulated gene expression occurred on the transcriptional level. Taken together, these results indicate that the pyrethroid permethrin is a PB-like inducer. Due to its superior potency in induction, permethrin appears as a useful substance for mechanistic studies to elucidate the mechanism of enzyme induction by phenobarbital.

Keywords: CYP2B1; Gene expression; Permethrin; PB-like inducer; PBREM; Xenobiotic metabolism

1. Introduction

Pyrethroids belong to the most widely used synthetic insecticidal agents. They are structurally derived from the naturally occurring pyrethrin esters and are well known for their high insecticidal potency combined with low mammalian toxicity. Their role in the etiology of diseases related to the patient's indoor environment remains to be elucidated. Generally, two types of pyrethroid insecticides leading to specific toxicity following high-dose exposure in mammals can be structurally differentiated: type I pyrethroids such as permethrin without a cyano group, which cause T syndrome (classified by tremor), and type II pyrethroids such as cypermethrin or fenvalerate bearing a cyano group in the α-position to the ester bond and causing CS syndrome (characterized by choreoathetosis and salivation) [1].

Induction of xenobiotic-metabolizing enzymes by xenobiotics is a common cellular defense mechanism against foreign compounds. The barbiturate PB is a classical inducer of several xenobiotic-metabolizing enzymes (e.g. CYP enzymes of the CYP2B and 3A subfamilies). In the development of insecticide-resistant insect strains, increased expression of CYPs plays an important role [2]. Therefore, the unspecific CYP inhibitor PBO is a common additive in commercially available pyrethroid and pyrethrin preparations. In pyrethroid-resistant housefly populations, an enhanced expression of CYP6D1, an isoform that is also inducible by PB, has been shown [3]. Little is known, however, about the influence of pyrethroid insecticides on

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Abbreviations: CYP, cytochrome P450; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MC, methylcholanthrene; PB, phenobarbital; PBO, piperonyl butoxide; PBREM, phenobarbital-responsive enhancer module; and PROD, pentoxyresorufin-*O*-depentylase.

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mammalian xenobiotic metabolism. In *in vivo* experiments in rats, high-dose exposure to pyrethroid insecticides resulted in a slight increase in total CYP content [4], but the isoforms involved in total CYP increase have not yet been defined. Recently, it has also been observed that a major metabolite of fenvalerate (fenvaleric acid) is capable of inducing hepatic xenobiotic metabolism in rats [5].

Chlorinated hydrocarbon pesticides, such as DDT (2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane) or dieldrin, that have a similar toxicity profile as pyrethroids cause a marked induction of CYP2B isoforms and to a lesser extent induction of CYP3A subfamily members in rats [6]. Since these pesticides induce the same pattern of enzymes in the rat as PB, they have been termed PB-like inducers [7]. Among the numerous mammalian enzymes known to be induced by PB (CYP2B subfamily, CYP3A, NADPH-dependent P450 reductase, UDP-glucuronosyltransferase isoforms etc.) [8], CYP2B1 is the isoform that is most prominently induced by PB in rat liver [7]. Transcriptional activation plays a pivotal role in CYP2B induction by PB. Recently, the PBREM situated in the distal promoter region of human, mouse, and rat *CYP2B* genes was identified as the major PB-responsive element [8–12].

With the parallel drawn from insect cytochromes that show inducibility dependent on PB or permethrin, the influence of pyrethroids on rat CYP2B1 expression in the presence and absence of PBO was examined. A primary rat hepatocyte culture system was employed in which inducibility of CYP2B1 by PB was retained [13–15].

In the present study, the influence of pyrethroids on the expression of the cytochrome P450 isoform 2B1 was studied in primary rat hepatocyte cultures in terms of mRNA levels, protein expression, and CYP2B-associated enzymatic activity. The effect of permethrin on *CYP2B1* promoter activation was further investigated in transiently transfected primary hepatocyte cultures.

2. Materials and methods

2.1. Chemicals

All chemicals used were of reagent grade and purchased from commercial suppliers. Collagenase and murine EGF were obtained from Roche Analytics. Hormones, pentoxyresorufin, resorufin, NADPH, dicumarol, 3-phenoxybenzaldehyde, and 3-phenoxybenzyl alcohol were purchased from Sigma, and [γ-^{32}P]ATP was supplied by DuPont NEN. Permethrin and cypermethrin were purchased from Riedel de Haen and fenvalerate from Dr. Ehrenstorfer GmbH. PBO was supplied by Fluka. pGL3 and pRL-TK reporter gene plasmids and Dual Luciferase Kits were obtained from Promega, Effectene transfection reagents were purchased from Qiagen, and endotoxin-free plasmid maxiprep kits from Macherey-Nagel.

2.2. Metabolite synthesis

3-Phenoxybenzoic acid was synthesized by oxidation of 3-phenoxybenzaldehyde using silver oxide. The purity of the product was verified using GC/MS analysis and was about 98%. 3-(2′,2′-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid was obtained by ester cleavage from permethrin performing alkaline hydrolysis, followed by column chromatography. The purity of the product was determined by GC/MS analysis and was about 90%.

2.3. Cell culture

Hepatocytes were isolated from male Wistar rats (180–220 g) by collagenase perfusion [16], resuspended in MX-82 medium [17] containing 10% fetal bovine serum, and seeded onto culture dishes, supplied by Nunc, coated with 4.1 μg/cm^2 type I collagen at a density of 8.6 $\times$ 10^4 cells/cm^2. Cultures were kept in a humidified atmosphere of 10% CO_2/90% air at 37°. After an initial attachment period of 3 hr, cells were washed with PBS and the medium replaced with serum-free MX-83 [17] lacking arginine but containing 1 μM insulin and 20 μM hydrocortisone. Hepatocytes were treated with pyrethroids (added to the medium from stock solutions in ethanol, max 1%) or with 16 nM EGF as indicated below. The medium was exchanged daily.

2.4. GC/MS analysis of permethrin metabolism in rat hepatocytes

Cultured rat hepatocytes were incubated with 100 μM permethrin for 3 hr and the supernatants subsequently subjected to liquid–liquid extraction with diethylether. Organic layers were evaporated to dryness, redissolved in ethanol, and analyzed by gas chromatography coupled with mass spectrometry according to the method of Pfleger *et al*., with slight modifications [18].

2.5. Northern blot analysis

Total RNA was isolated by guanidinium thiocyanate–phenol–chloroform extraction [19] and subjected to electrophoresis through formaldehyde/agarose gels (20 μg RNA/lane). To control equal loading, ethidium bromide gel staining was evaluated. Subsequently, RNA was transferred to Hybond N nylon membrane (Amersham) by capillary transfer and hybridized to specific antisense oligonucleotide probes, synthesized by Genaxis GmbH. These probes were for rat CYP2B1 [20], for rat CYP3A1 with the sequence 5′-CTT AAA CAC GGA GCC ATC ATC-3′ from exon 1 of the rat *CYP3A1* gene, for rat GAPDH with the sequence 5′-CAG GAT GCA TTG CTG ACA ATC TTG A-3′ from the rat *GAPDH* gene, and for rat β-actin with the sequence 5′-GAC TTC CTG TAA CCA TCT ATG CCG TG-3′ from the rat *β-actin* gene end-labeled by T4 polynucleotide kinase (Promega, distributed by Serva) using [γ-^{32}P]ATP

(DuPont NEN) according to Omiecinski [20]. Hybridization was performed at 38°. Blots were washed up to a stringency of 0.1 × standard saline citrate buffer (SSC) containing 0.1% SDS at 38°.

For the detection of CYP1A1 mRNA, cDNA fragments homologous to rat CYP1A1 [21] were excised from the plasmid vector pSV450IA1 and labeled with [α-^{32}P]dCTP by random oligonucleotide priming [22,23]. Hybridization was performed at 45°. Blots were washed up to a stringency of 0.1 × SSC/0.1% SDS at 50°.

2.6. Western blot analysis

Microsomal protein was obtained by differential centrifugation using a modification of the method according to Dallner [24]. The protein content of the samples was determined according to the method described by Lowry *et al*. [25]. Samples of 10 μg protein/lane were subjected to SDS–PAGE through 10% gels [26] and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by semidry blotting [27] using a modification of the anode buffer (48 mM Tris–HCl, 39 mM glycine, 0.38% w/v SDS, 15% v/v MeOH). Immunodetection of CYP2B-related protein was performed using a polyclonal primary antibody (Daiichi Pure Chemical Co. Ltd., distributed by NatuTec) and a secondary peroxidase-conjugated antibody (Sigma). The microsomal fraction from the liver of a rat treated with PB (supplied by Gentest Corporation and distributed by NatuTec) served as CYP2B protein reference. Visualization of CYP2B bands was achieved by enhanced chemoluminescence, employing reagents (the peroxidase substrate luminol and Hyperfilm ECL) supplied by Amersham.

2.7. Enzymatic assay procedures

PROD activities were measured by modification of the method described by Lubet *et al*. [28]. In brief, cultivated hepatocytes (approximately 10^7 cells) were scraped from the culture dishes with a rubber policeman, suspended in 2 mL TES buffer (containing 10 mM Tris–HCl, 1 mM EDTA, and 250 mM sucrose), and homogenized by sonication at cycle 1 and amplitude 100% for 30 sec using a UP 50H sonifier (Dr. Hielscher). Lysates obtained were then centrifuged at 9000 × *g* for 5 min. PROD activities were measured at room temperature in a final volume of 2 mL PBS containing 500 μL of supernatant (S9 mix, supernatant of 9000 × *g* centrifugation) in the presence of 10 μM substrate (pentoxyresorufin) and 10 μM dicumarol in order to inhibit diaphorase activity. Reactions were initiated by addition of 5 μL of 50 mM NADPH. The formation of resorufin was monitored spectrofluorimetrically using an RF-50001PC (Shimadzu), with the excitation wavelength being set at 530 nm and the emission wavelength at 580 nm. The reaction rate was determined by external calibration with solutions of known resorufin concentrations. The enzyme activities were standardized on the basis of protein contents, determined according to Lowry *et al*. [25] with BSA as a standard.

2.8. CYP2B1 promoter activity assay

2.8.1. Luciferase reporter gene construct

A 2677-bp fragment of the 5′-flanking promoter region of the rat *CYP2B1* gene was amplified by polymerase chain reaction (PCR) from genomic rat hepatocyte DNA using the following primer sites according to the published promoter sequence [29]: bases −2648 to −2623 (forward), 29 to 4 (reversed). The primers comprised a 5′-Nhe I recognition site and a three-base overhang resulting in 35-mers. The obtained PCR fragment was digested with Nhe I and ligated into the Nhe I site of the pGL3 basic vector. The sequence of the resulting promoter reporter gene construct designated as pGL3C2B1 was verified by sequence analysis using standard sequencing primers of the pGL3 plasmid and internal primers of the insert. Endotoxin-free maxi-prep kits (Qiagen) were used for plasmid purification.

2.8.2. Transient transfection

Rat hepatocytes in MX-82 medium containing 10% FBS (fetal bovine serum) were seeded onto 35-mm dishes (6-well plates). Four hours after seeding, the medium was replaced by 1 mL fresh MX-83 medium/well without FBS. Twenty-four hours after seeding, the hepatocytes were transiently transfected using 0.6 μg of pGL3C2B1, 0.6 μg of pRL-TK, 15 μL Effectene reagent, 9.5 μL Enhancer reagent (Qiagen), and an additional 0.6 mL of MX-83 medium according to the manufacturer's manual. Six hours later, the medium was replaced with 1.6 mL fresh medium containing the indicated concentrations of inducers (PB, permethrin). The medium was replaced by fresh medium 24 hr posttransfection. After 48 hr of treatment with inducers, the hepatocytes were washed with PBS and lysed by 150 μL Passive Lysis Buffer (Dual Luciferase™ Reporter Assay, Promega). Firefly and *Renilla* luciferase activity were measured in 20 μL hepatocyte lysate using a Berthold Lumat LB 9501 luminometer according to the Dual Luciferase™ Reporter Assay protocol.

3. Results

3.1. Induction of CYP2B1 mRNA by permethrin, permethrin plus piperonyl butoxide, cypermethrin, or fenvalerate

Primary rat hepatocytes were cultured in the absence or presence of the type I pyrethroid permethrin, a mixture of permethrin and PBO, or type II pyrethroids (cypermethrin and fenvalerate) for 1 to 3 days. All pyrethroid insecticides led to a significant induction of CYP2B1 mRNA which increased with culture duration and was maximal with permethrin after 3 days of culture (Fig. 1; kinetic data for

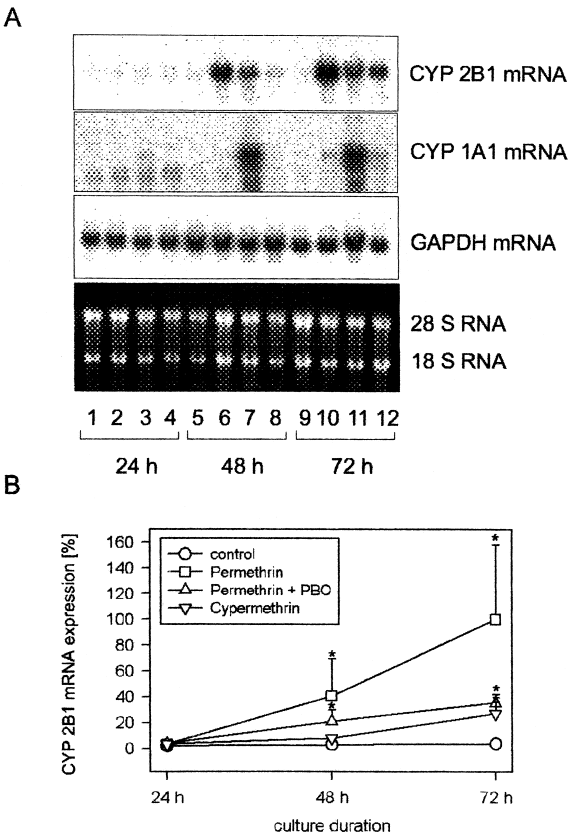

Fig. 1. Time-dependent CYP2B1 and CYP1A1 mRNA expression under the influence of pyrethroids in primary rat hepatocyte cultures. Hepatocytes were cultured in the presence of 1% EtOH (vehicle), permethrin, permethrin + PBO, and cypermethrin, all at concentrations of 100 μM, for 1 to 3 days. mRNA levels were determined by Northern blot analysis as depicted in (A) (representative blot). Subsequently, the blot was rehybridized to a GAPDH-specific oligonucleotide probe as described in the experimental section. Ethidium bromide staining of corresponding agarose gels indicated equal loading of lanes (bottom panel). Lanes represent: 1,5,9: control (1% ethanol); 2,6,10: permethrin; 3,7,11: permethrin + PBO; 4,8,12: cypermethrin. Hybridization was quantified by phosphorimaging analysis. Data in (B) represent mean values ± SEM of three independent experiments. CYP2B1 mRNA expression on day 3 under permethrin was set to 100%. *Significant difference between control and inducer. ($P < 0.05$, Student's *t*-test).

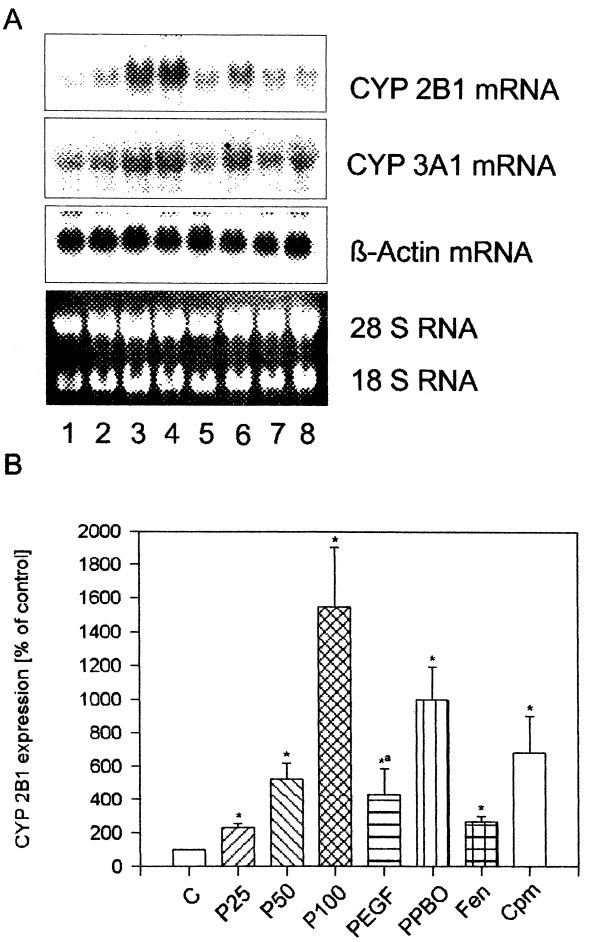

Fig. 2. CYP2B1 and CYP3A1 mRNA expression under the influence of various permethrin concentrations (P), permethrin + EGF (PEGF), permethrin + PBO (PPBO), fenvalerate (Fen), and cypermethrin (Cpm). Lanes in (A) represent 1: control; 2: 25 μM; 3: 50 μM; 4: 100 μM permethrin; 5: 100 μM permethrin + 16 nM EGF; 6: 100 μM permethrin + 100 μM PBO; 7: 100 μM fenvalerate; 8: 100 μM cypermethrin. Panel B shows mean values ± SEM from 10 or more independent experiments of CYP2B1 mRNA expression as % of control. Control levels were set to 100%. *Significant difference between control and expression in the presence of an inducer; *,[a]significant difference between mRNA expression under permethrin and expression under permethrin in the presence of EGF ($P < 0.05$, Student's *t*-test).

fenvalerate not shown). Although CYP1A1 mRNA levels appeared to be slightly elevated by permethrin and cypermethrin in some experiments (Fig. 1A), this elevation did not reach statistical significance. However, CYP1A1 mRNA was markedly induced by the mixture of permethrin and PBO (Fig. 1A). PBO alone was capable of inducing CYP1A1 mRNA expression to the same extent as the mixture of permethrin and PBO (data not shown).

CYP2B1 mRNA induction was demonstrated to be dose-dependent (data shown for permethrin in Fig. 2) in all cases up to a pyrethroid concentration of 100 μM, which represents the limit of solubility. mRNA expression of CYP3A1, a CYP isoform also inducible by phenobarbital, was increased 2- to 3-fold by permethrin (Fig. 2A). In the presence of PBO, an inhibitor of CYP activity, permethrin-dependent CYP2B1 mRNA induction occurred to a lesser extent than in the absence of PBO. Though the difference was not statistically significant (Fig. 2), the question was raised as to whether permethrin metabolites might be involved in CYP2B1 induction.

Pyrethroids are well known to be extensively metabolized in rats *in vivo* and by microsomal preparations *in vitro* including carboxyl esterases and CYP-dependent monooxygenases (isoforms not analyzed in detail), but pyrethroid metabolism in cultivated rat hepatocytes had not been investigated before. For metabolite analysis, rat hepatocytes cultivated for three days were incubated in the presence of permethrin and the supernatants analyzed by gas chromatography coupled with mass spectrometry as described in

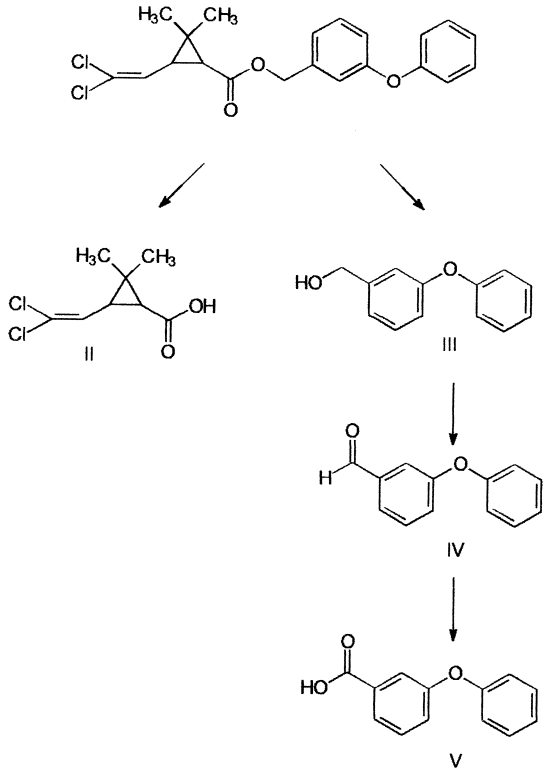

Fig. 3. Metabolites of permethrin in rat hepatocyte cultures identified by GC/MS analysis. I = permethrin; II = 3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; III = 3-phenoxybenzyl alcohol; IV = 3-phenoxybenzaldehyde; V = 3-phenoxybenzoic acid. Formation of the identified substances is followed by further oxidation and conjugation.

section 2. After 3 hr of incubation, about 40% of the applied permethrin had disappeared from the medium. The metabolites found were the products of ester cleavage and oxidation of the resulting alcohol moiety shown in Fig. 3. 3-Phenoxybenzoic acid (V) was the most abundant metabolite with concentrations of about 15 μM in the medium, as determined by comparison to peak areas of known amounts of 3-phenoxybenzoic acid in the total ion chromatogram. In contrast, 3-phenoxybenzyl alcohol was present at very low concentrations. These metabolites are in accordance with those found in the rat *in vivo* and in microsomal preparations [30,31]. To investigate whether pyrethroid metabolites might be involved in the induction of CYP2B1 mRNA expression, cells were incubated with the major permethrin metabolites shown in Fig. 3: 3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (II), 3-phenoxybenzyl alcohol (III), and 3-phenoxybenzoic acid (V). However, the products of metabolic ester cleavage and subsequent oxidation (II, III, and V) as shown in Fig. 3 did not lead to a marked induction of CYP2B1 mRNA (Fig. 4, data not shown for 3-(2′,2′ dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid). Thus, CYP2B1 mRNA induction by permethrin was not mediated by the permethrin metabolites examined.

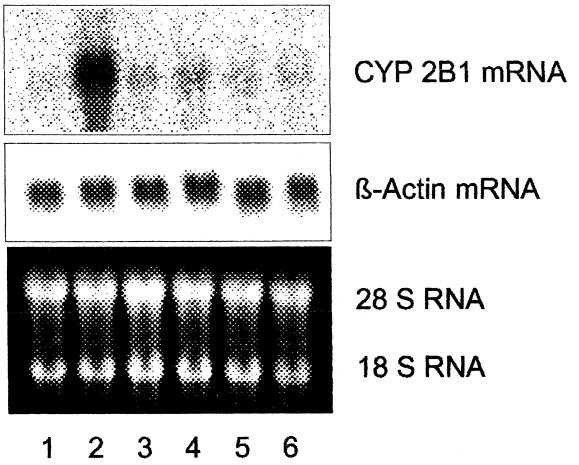

Fig. 4. Influence of major pyrethroid metabolites on CYP2B1 mRNA expression in primary rat hepatocytes cultured for 3 days. Lanes represent: 1: control; 2: 100 μM permethrin; 3: 50 μM 3-phenoxybenzyl alcohol; 4: 100 μM 3-phenoxybenzyl alcohol; 5: 50 μM 3-phenoxybenzoic acid; 6: 100 μM 3-phenoxybenzoic acid. The blot was rehybridized to a β-actin-specific probe as described in the experimental section.

3.2. Increase in CYP2B protein in the presence of increasing permethrin concentrations

After 72 hr of culture, microsomal protein was obtained from hepatocytes and subjected to SDS–PAGE. For immunodetection of CYP2B protein, a polyclonal antibody against rat CYP2B was used. In the absence of permethrin, CYP2B protein was barely detectable. In the presence of 25 to 100 μM permethrin, CYP2B protein expression was markedly enhanced up to 5- to 7-fold (Fig. 5 shows a representative Western blot analysis of three independently performed experiments). In contrast to mRNA induction (an increase in mRNA expression was observed up to 100 μM (Fig. 2), a maximal CYP2B protein induction was already observed at the lowest concentration examined (25 μM, Fig. 5). An increase in CYP2B protein expression was also observed in hepatocytes treated with 100 μM cypermethrin or 100 μM fenvalerate, amounting to approximately 2-fold induction (data not shown).

3.3. Modulation of CYP2B-associated enzyme activity

To investigate whether induction of CYP2B protein by pyrethroids would result in enhanced CYP2B-associated enzyme activity, PROD reactions were performed with S9 mixtures obtained from hepatocytes cultured for three days in the presence of increasing concentrations of permethrin and permethrin plus piperonyl butoxide, cypermethrin, and fenvalerate. PROD activity was substantially enhanced in all preparations from hepatocytes treated with pyrethroids, particularly in cells exposed to permethrin (approx. 15-fold). A permethrin concentration of 25 μM in the culture medium already led to a maximal enhancement of PROD activity (Table 1).

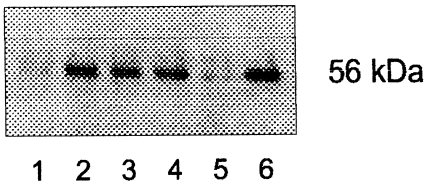

Fig. 5. Influence of permethrin in increasing concentrations on the expression of CYP2B protein and repression of permethrin-dependent CYP2B induction by EGF in primary rat hepatocytes cultured for 3 days. Samples containing 10 μg protein were subjected to Western blot analysis as outlined in the experimental section. Lanes represent: 1: control; 2: 25 μM, 3: 50 μM; 4: 100 μM permethrin; 5: 100 μM permethrin + 16 nM EGF; 6: CYP2B reference (microsomes of PB-induced rat liver).

3.4. Inhibition of induction by EGF

Cytokines such as EGF or TNF-α (tumor necrosis factor alpha) are known inhibitors of induction of several xenobiotic-metabolizing cytochromes P 450. In particular, PB-dependent induction of CYP2B1 has been shown to be repressed by EGF [32]. To investigate whether EGF might mediate a repression of induction by permethrin, cells were cultured in the presence of 16 nM EGF for up to 3 days. Substantial inhibition of induction on the level of mRNA to about 28% of control induction was observed (Fig. 2). In Western blot analyses and in functional assays, a complete repression of the induction of CYP2B protein and related activities by EGF to levels corresponding to the controls was demonstrated, as shown in Fig. 5 as well as in Table 1. Furthermore, EGF also repressed permethrin-dependent induction of CYP3A1 mRNA (Fig. 2A).

3.5. Stimulation of CYP2B1 promoter activity by permethrin

To examine whether the induction of CYP2B1 by permethrin might be based on transcriptional activation, primary rat hepatocytes were transiently transfected with a firefly luciferase reporter gene plasmid that contained the complete 5′-flanking region of the *CYP2B1* promoter up to approximately −2.7 kb [29] and comprised the PBREM. Firefly luciferase activity was measured in cell lysates obtained 48 hr after transfection and standardized according to *Renilla* luciferase activity resulting from the co-transfected pRL-TK plasmid. Treatment of the transfected cells with 50 μM permethrin resulted in a 40-fold stimulation of firefly luciferase activity, while incubation with 1.5 mM PB led to an approximately 4-fold induction of luciferase activity, as shown in Fig. 6B. The superior potency of permethrin as compared to PB in inducing *CYP2B1* gene expression was also observed on the mRNA level, as demonstrated in the Northern blot analysis shown in Fig. 6A.

Table 1.
PROD activities in hepatocytes treated with pyrethroids

Treatment	PROD activity [nmol/mg/min]
Control	0.06 ± 0.03
25 μM Permethrin	1.04 ± 0.19*
50 μM Permethrin	0.96 ± 0.02*
100 μM Permethrin	0.82 ± 0.15*
100 μM Permethrin + 16 nM EGF	0.14 ± 0.05**
100 μM Permethrin + 100 μM PBO	0.35 ± 0.02[a],*
100 μM Fenvalerate	0.34 ± 0.02[a],*
100 μM Cypermethrin	0.29 ± 0.03[a],*

Influence of pyrethroids and EGF on CYP2B-associated PROD activity in S9 preparations of primary rat hepatocytes cultured for 3 days in the presence of pyrethroids and other modulators. Data represent mean values of three or more independent experiments ± SEM.

[a] Mean values ± SEM of single experiments performed in triplicate.

* Significant difference between control and inducer-treated cultures ($P < 0.05$ Student's *t*-test).

** Significant difference between permethrin- and permethrin + EGF-treated cultures ($P < 0.05$ Student's *t*-test).

4. Discussion

Exposure to pyrethroid insecticides has been shown to lead to the development of resistant insect strains [2]. Resistance against pyrethroids in insects is frequently associated with the induction of CYP isoforms that also exhibit phenobarbital-dependent inducibility. It is known that organochlorine insecticides such as DDT that have a similar mechanism of toxicity (inhibition of voltage-gated sodium channels) towards target organisms such as pyrethroids [33, 34] are capable of inducing mammalian cytochromes P450 in a PB-like manner [35]. However, the impact of pyrethroids on xenobiotic-metabolizing enzymes in mammalian organisms is still highly unexplored.

To elucidate the possible influence of pyrethroids on mammalian xenobiotic-metabolizing enzymes (CYP2B1, CYP1A1, CYP3A1), a primary rat hepatocyte culture system was employed in which hepatocyte-specific inducibility of CYP2B1 by PB was retained [13–15]. All pyrethroids used for the experiments (permethrin, cypermethrin, and fenvalerate) led to a marked induction of CYP2B1 mRNA, CYP2B protein, and CYP2B-associated enzyme activity. The most pronounced effect on all tested parameters was achieved with the type I pyrethroid permethrin. The induction followed a kinetic profile similar to that of CYP2B1 induction by PB in primary rat hepatocyte cultures, where maximal induction was observed within 3 days of culture [13–15]. As demonstrated in Fig. 6A, CYP2B1 mRNA induction by 50 μM permethrin exceeded CYP2B1 induction by PB at 1.5 mM, the PB concentration shown to elicit maximal CYP2B1 mRNA induction in this culture system [13]. In the present study, the induction of CYP2B1 mRNA by pyrethroids was dose-dependent in a range of 25–100 μM, while CYP2B protein as well as CYP2B-associated enzyme activity were not dose-dependent in the examined

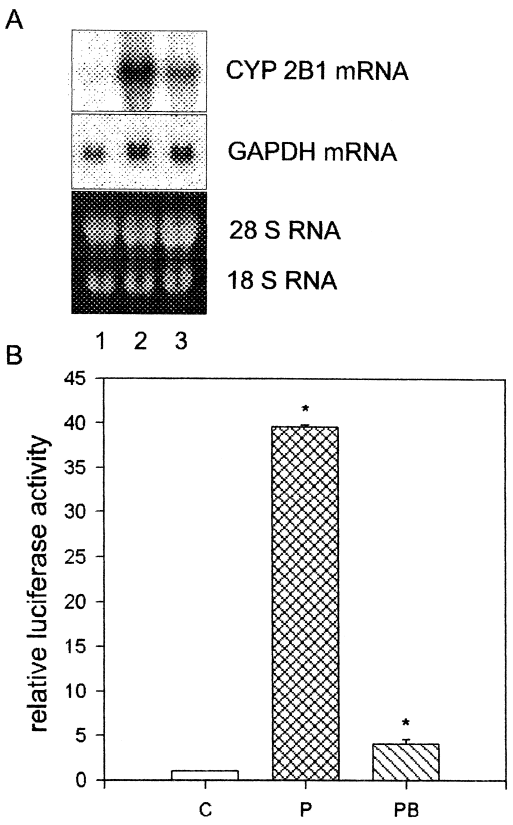

Fig. 6. (A) Direct comparison of the influence of 50 μM permethrin (2) and 1.5 mM PB (3) versus control (1) on CYP2B1 mRNA expression in primary rat hepatocytes, cultured for 3 days (Northern blot analysis). (B) Relative luciferase activity in the absence of an inducer (C) in the presence of 50 μM permethrin (P) or 1.5 mM phenobarbital (PB), respectively. Primary rat hepatocytes were transfected with the reporter gene construct pGL3C2B1 24 hr after seeding and incubated with permethrin or PB for 48 hr following transfection. The transfection efficiency was evaluated by cotransfection with a *Renilla* luciferase construct (pRL-TK) and by standardization of firefly luciferase activities according to the corresponding *Renilla* luciferase activities. Data represent mean values ± SEM of a representative experiment performed in triplicate that was representative of three independent hepatocyte preparations. Control levels were set to 1. *Significant difference between control and inducer-treated cultures ($P < 0.05$ Student's *t*-test).

concentration range. It is possible that permethrin, in addition to induction of mRNA, already acts on the basis of protein stabilization at the lowest concentration tested. Interestingly, modulation of gene expression by permethrin was not confined to CYP2B1, but also affected the PB-inducible CYP3A1, resulting in a 2- to 3-fold induction. This supports the notion that permethrin, like PB, induces a multiplicity of genes in the liver, and thus markedly affects mammalian xenobiotic metabolism.

'Crossover' induction of MC-inducible CYP isoforms by PB *in vitro* has been reported [36]. Induction of MC-inducible CYP1A1 mRNA by permethrin or cypermethrin alone was not detected to a statistically significant extent, but the mixture of permethrin and PBO resulted in a very strong increase in CYP1A1 mRNA levels, indicating that CYP1A1 was induced by PBO. This result is in accordance with the fact that PBO is capable of inducing CYP1A-associated ethoxyresorufin-*O*-deethylase activity in rats *in vivo* [37]. These data indicate that exposure to insecticide preparations containing pyrethroids and PBO may affect the hepatocytes' capacity for processing xenobiotics. The present study demonstrates that the mixture of permethrin and PBO induces both PB- and MC-inducible CYP isoforms that are involved not only in detoxification of xenobiotics, but partly in the conversion of precarcinogenic substances to ultimate carcinogens.

When cells were incubated with combinations of permethrin and PBO, the unspecific inhibitor of CYP activity, the induction of CYP2B1 in all experiments occurred to a lesser extent than with permethrin alone, though the difference was not statistically significant. Nevertheless, the question was raised as to whether permethrin metabolites might be involved in induction. For this purpose, permethrin metabolism was studied in cultured rat hepatocytes. The metabolites found in the model system used here were the same as in other *in vitro* and *in vivo* model systems [31,32]. All examined permethrin metabolites (3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid and 3-phenoxybenzoic acid) did not mediate induction of CYP2B1. From Fig. 4, 3-phenoxybenzyl alcohol (III) appeared to very weakly induce CYP2B1 mRNA at the concentration of 100 μM. However, since it was one of the least abundant metabolites formed following hepatocyte incubation, reflecting its further metabolism to 3-phenoxybenzoic acid, it is considered not to contribute to a major extent to the observed induction of CYP2B1 mRNA in the model system used. These data support the notion that the unchanged molecular structure of permethrin is crucial for induction, which appears to be another parallel to phenobarbital-dependent enzyme induction: *p*-hydroxy phenobarbital, a major hydroxylated metabolite of phenobarbital, did not lead to a comparable induction of CYP2B1 mRNA *in vivo* [38]. However, the involvement of unidentified or conjugated metabolites in the process of pyrethroid-dependent enzyme induction cannot be completely ruled out, as fenvaleric acid, a major fenvalerate metabolite, has been reported to be an inducer of several mammalian xenobiotic-metabolizing enzymes (CYP2B activity and peroxisome proliferation) [5].

PB-dependent CYP2B1 induction was shown to be repressed by EGF in primary hepatocytes [14,15]. Repression of the induction of permethrin-inducible CYP2B1 by EGF was substantial on the mRNA, protein, and functional levels. Again, this effect is reminiscent of repression of PB-dependent CYP2B1 induction. However, MC-dependent CYP1A1 induction is subject to repression by EGF as well [13], so this effect does not appear to be confined to PB-inducible isoforms.

A common feature of many known PB-type inducers is their relatively low potency in comparison to MC-type inducers, which are active in the nanomolar concentration range. This makes the identification of receptors by conven-

tional receptor assay procedures difficult [39]. Using PB as an inducer *in vitro* in the currently employed hepatocyte culture system, concentrations in the millimolar range are required to achieve maximal induction [14]. In contrast, induction of CYP2B1 mRNA exceeding induction by 1.5 mM PB was achieved with 50 μM permethrin in the culture medium. Thus pyrethroids, especially permethrin, appear as highly potent model substances for mechanistic studies on CYP2B induction in the rat, like 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) that is known to be a potent PB-type inducer in the mouse [40].

To investigate whether CYP2B1 mRNA induction might be based on transcriptional activation, a *CYP2B1* promoter assay was performed. The reporter gene construct used to transfect primary rat hepatocytes comprised the firefly luciferase gene driven by the *CYP2B1* promoter, which contained the PBREM. A 40-fold enhancement of reporter gene activity in hepatocytes treated with 50 μM permethrin identified permethrin as a highly potent inducer eliciting marked activation of the *CYP2B1* promoter. In contrast, 1.5 mM PB elicited only a 4-fold promoter activation. These results support the conclusion that transcriptional activation is of major importance in induction of CYP2B1 expression in hepatocyte cultures by permethrin, and again, demonstrate the superior potency of permethrin as compared to PB in activating *CYP2B1* gene expression.

In summary, the present study indicates that exposure to mixtures of permethrin and PBO, both constituents of commercially available pyrethroid insecticide preparations, leads to induction of several xenobiotic-metabolizing enzymes (induction of CYP2B1 and CYP3A1 by permethrin and induction of CYP1A1 by PBO) and thus alters the hepatocytes' capacity to process xenobiotics. Furthermore, several parallel features of CYP induction elicited by PB and permethrin indicate that induction of CYP2B1 by PB and permethrin might occur via common molecular mechanisms.

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