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Characterization of human cytochrome P450 induction by pesticides

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ABSTRACT

Pesticides are a large group of structurally diverse toxic chemicals. The toxicity may be modified by cytochrome P450 (CYP) enzyme activity. In the current study, we have investigated effects and mechanisms of 24 structurally varying pesticides on human CYP expression. Many pesticides were found to efficiently activate human pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR). Out of the 24 compounds tested, 14 increased PXR- and 15 CAR-mediated luciferase activities at least 2-fold. While PXR was predominantly activated by pyrethroids, CAR was, in addition to pyrethroids, well activated by organophosphates and several carbamates. Induction of CYP mRNAs and catalytic activities was studied in the metabolically competent, human derived HepaRG cell line. CYP3A4 mRNA was induced most powerfully by pyrethroids; 50 μM cypermethrin increased CYP3A4 mRNA 35-fold. CYP2B6 was induced fairly equally by organophosphate, carbamate and pyrethroid compounds. Induction of CYP3A4 and CYP2B6 by these compound classes paralleled their effects on PXR and CAR. The urea herbicide diuron and the triazine herbicide atrazine induced CYP2B6 mRNA more than 10-fold, but did not activate CAR indicating that some pesticides may induce CYP2B6 via CAR-independent mechanisms. CYP catalyzed activities were induced much less than the corresponding mRNAs. At least in some cases, this is probably due to significant inhibition of CYP enzymes by the studied pesticides. Compared with human CAR activation and CYP2B6 expression, pesticides had much less effect on mouse CAR and CYP2B10 mRNA. Altogether, pesticides were found to be powerful human CYP inducers acting through both PXR and CAR.

1. Introduction

Pestidices are a widely used, structurally diverse group of chemicals designed to kill or repel pests such as insects, weeds, or microbes, but they also have potential for toxic effects on human health. Humans are exposed to pesticides via inhalation, dermal contact and ingestion due to occupational exposure, in the environment and through contaminated food (Gilden et al., 2010). Pesticides are suspected to have harmful neurological, immunological, reproductive and cancerous health effects (Gilden et al., 2010). For example, organophosphate (OPs) and carbamates insecticides are synaptic poisons, which bind to and inhibit acetylcholinesterase and epidemiological studies suggest that exposure to pesticides may be associated with Parkinson disease among humans (Wirdefeldt et al., 2011).

Many pesticides are subjected to metabolic biotransformation by human hepatic cytochrome P450 (CYP) enzymes (Abass et al., 2012). This event is generally considered as a detoxification process; however, in several cases CYP enzymes catalyze bioactivation of pesticides. For example, more toxic metabolites arise in the case of desulfuration of organophosphorus insecticides (Poet et al., 2003; Buratti et al., 2003, 2005; Mutch and Williams, 2006; Abass et al., 2007a; Foxenberg et al., 2007; Leoni et al., 2008) and metabolism of carbamate insecticides (Usmani et al., 2004; Abass et al., 2009a, 2010; Croom et al., 2010).

Abbreviations: CYP, Cytochrome P450; PXR, Pregnane X receptor; CAR, Constitutive androstane receptor; XREM, Xenobiotic reponsive enhancer module; LBDs, Ligand-binding domains; AhR, Aryl hydrocarbon receptor; TCPOBOP, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene; TMPP, Tri-p-methylphenylphosphate; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; OPs, Organophosphate; LC/MS–MS, Liquid chromatography–tandem mass spectrometry; RT-qPCR, Quantitative real-time reverse transcription polymerase chain reaction; RIF, Rifampicin; PB, Phenobarbital; DEX, Dexamethasone; DMSO, Dimethyl sulfoxide.

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Interestingly, several pesticides among OPs, pyrethroids, carbamates and urea compounds have been reported to induce CYP expression (de Sousa et al., 1997; Yang et al., 2009; Lemaire et al., 2004; Takeuchi et al., 2008; Rudzok et al., 2009; Das et al., 2008a, 2008b, 2006; Casabar et al., 2010). Pesticides can induce the CYP involved in their own metabolism as well as the metabolism of other compounds that are substrates for the same CYP enzymes. Induction of CYP enzymes alters the balance between detoxification and activation and in some cases this may lead to enhanced toxicity. For example, a classical CYP inducer phenobarbital potentiated neurotoxic effect of deltamethrin (Dayal et al., 2003).

The xenobiotic-sensing nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are major regulators of CYP gene expression (Pelkonen et al., 2008). Many pesticides have been suggested to be PXR agonists. A recent study reported human or mouse PXR-mediated transcriptional activation by 28 out of 56 tested OP pesticides. All the tested pyrethroids, e.g. cypermethrin, deltamethrin, permethrin, fenvalerate and cyhalothrin, were PXR agonists. Moreover, metalaxyl, diuron and several carbamate-type pesticides including pyributicarb, esprocarb, thiobencarb and dimepiperate activated human and mouse PXR (Kojima et al., 2011). CAR is also activated by some pesticides such as cypermethrin, alachlor, imazalil and chlorpyrifos, but this has been studied much less than PXR (Wyde et al., 2003; Baldwin and Roling, 2009).

Activation of xenobiotic-sensing nuclear receptors can be measured with several high throughput in vitro methods (Pelkonen et al., 2008). However, observed activation of a nuclear receptor in a screening assay does not always necessarily indicate significant induction of the CYP enzyme activities. This may be due to rapid metabolism of the inducing agent, simultaneous inhibition of the CYP activity or other reasons (Sinz et al., 2006). In the present screening study we have systematically analysed the effect of 24 structurally diverse pesticides (Suppl. Table 1) on human PXR and CAR activation. The nuclear receptor activation was then compared to actual induction of the major CYP mRNAs as well as the CYP-mediated catalytic activities in the metabolically competent human HepaRG cell line. Furthermore, we compared species differences in the induction of mouse and human CYPs and the CAR activation. Most pesticides were found to be potent inducers of a wide range of CYPs. Important differences in the activation of PXR and CAR and subsequent CYP induction by different chemical classes were also observed.

2. Materials and methods

2.1. Chemicals

Pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and ChemService (West Chester, PA). Midazolam was a kind gift from F. Hoffmann La Roche (Basel, Switzerland) and omeprazole from Astra Zeneca (Mölndal, Sweden). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from the Sigma Chemical Company (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with the Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP grade (ultra pure, 18.2 MΩ).

2.2. Cell cultures and isolation of mouse hepatocytes

HepaRG cells were obtained from Biopredic Ltd. (Rennes, France). The procedures for plating and maintaining HepaRG cells were carried out as described previously in detail (Aninat et al., 2006). Cell cultures were maintained at +37 °C in humidified air incubator with 5% CO_2. The differentiated HepaRG cells in 24-well plates, seeded at the density of 0.05 million cells per well, were cultured either in Williams' E medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine and 5×10^{-5} M hydrocortisone hemisuccinate, or in differentiation medium (DIFF) corresponding to the Williams' E medium supplemented with 2% of DMSO. HepG2 and C3A hepatoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Gaithersburg, MD) with 10% foetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen).

Mouse hepatocytes were isolated from male DBA/2 (OlaHsd) mice (Harlan, Holland), aged 8–10 weeks, by perfusion with collagenase solution as described previously (Arpiainen et al., 2008). After filtration and centrifugation, the isolated hepatocytes were dispersed in Williams' E medium containing 20 ng/ml dexamethasone, ITS (5 mg/l insulin, 5 mg/l transferrin, 5 μg/l sodium selenite), 10 μg/ml gentamicin, 1% L-glutamine, and 10% decomplemented fetal calf serum at a density of 1.8×10^6 cells/60-mm uncoated culture dish (Corning, Palo Alto, CA), and 3×10^5 cells/well on 12-well plates (FALCON Polystyrene Cell Culture Dish, BD Biosciences, San Jose, CA). The cultures were maintained at +37 °C in 5% CO_2 in a humidifier incubator. After 2 h of incubation, the medium was replaced with serum-free Williams' E medium. The cultures were maintained for additional 24 h before treatment with tested compounds. Animal experiments were reviewed and approved by the national Animal Experiment Board.

2.3. Lactate dehydrogenase assay

Cytotoxicity was quantified by the activity of the lactate dehydrogenase (LDH) released from the cytosol of damaged cells into the medium of the cultures. The activity was determined using a Cytotoxicity Detection kit (ClonTech, USA) in a ELISA plate reader (Dynex MRX TC Revelation ELISA reader), following the manufacturer's instructions. The assay is based on the reduction of NAD^+ to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. Next, the enzyme diaphorase transfers H/H^+ from $NADH/H^+$ to tetrazolium salt INT, which is reduced to formazan dye, which shows a broad absorption maximum at about 500 nm. The results are expressed as a percentage of total activity released with 1% Triton X-100 in assay medium.

2.4. Activation of human PXR

Approximately 2×10^5 HepG2 cells were seeded per well on 48-well Plates 24 h before transient transfection of cells using Tfx-20 (Promega). Cells were transfected according to manufacturer's protocol, in Opti-MEM I medium (Invitrogen), with 0.35 μg of pGL3-XREM, 0.032 μg of PXR vector, and 0.032 μg of pRL-TK for normalization of transfection efficiency. Expression vector for human PXR (pSG5-hPXR) was a kind gift from Dr. Steven Kliewer (University of Texas Southwestern Medical Center, USA). pGL3-XREM luciferase reporter plasmid is under the control of PXR responsive XREM sequence from CYP3A4 promoter (CYP3A4 5′ −7836 to −7604 in front of TK promoter). On the following day, medium was replaced with fresh medium together with vehicle DMSO (0.5%, v/v), rifampicin (10 μM) or test compounds (10 μM and 50 μM). Forty-eight hours after chemical induction, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) with Labsystems luminometer (Life Science International). Data shown in each figure are presented as mean ± SD. Each figure is representative of at least 5–6 independent replicate experiments.

2.5. Activation of mouse and human CAR

C3A hepatoma cells were cultured in 48-well plates to reach 50% confluence in phenol red-free DMEM (Invitrogen, Gaithersburg, MD) complemented with 10% FBS (BioWhittaker, Cambrex, Belgium), 1% L-glutamine (Euroclone, Pero (Milano), Italy) and antibiotics. The cells were transfected with CMV-driven plasmids expressing GAL4 fusions of mouse and human CAR ligand-binding domains (LBDs) (450 ng/well), GAL4-responsive UAS4-tk-luciferase reporter (300 ng/well) and the transfection control reporter pCMVβ (600 ng/well) with the calcium phosphate method as described (Mäkinen et al., 2003; Jyrkkärinne et al., 2005). After transfection for 5 h, the medium was replaced with fresh DMEM including either the vehicle DMSO (0.1%, v/v), positive control substances [TCPOBOP (1 μM) and chlorpromazine (10 μM) for mouse CAR (Mäkinen et al., 2003); TMPP (10 μM (Jyrkkärinne et al., 2005)) and FL81 (10 μM (Küblbeck et al., 2011)) for human CAR; tested chemicals (10 or 50 μM) and 5% delipidated serum (HyClone, Logan, UT) in lieu of 10% FBS]. After 24 h of exposure, the cells were lysed and the luciferase and β-galactosidase activities (Mäkinen et al., 2003; Jyrkkärinne et al., 2005) were measured from 20 μl of the cell lysate using the Victor™ (Jyrkkärinne et al., 2005) multiplate reader (PerkinElmer Wallac, Turku, Finland). All luciferase activities were normalized to β-galactosidase activities and the results are expressed as mean ± standard deviation (SD) of at least three independent experiments.

2.6. cDNA synthesis and real-time PCR

HepaRG and mouse primary hepatocytes were exposed to vehicle DMSO (0.5%, v/v), model chemical inducers or test compounds for 24 h before isolation of RNA using NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Complementary DNA synthesis from total RNA (1 μg) was done using random hexamer primers (Roche Diagnostics, Mannheim, Germany) in a reaction driven by M-MLV reverse transcriptase (Promega). RNA was protected with Stop RNase Inhibitor (5 Prime, Hamburg, Germany). Relative gene expression levels were quantitated by the comparative Ct (ΔΔCt) method, using FastStart Universal SYBR Green Master mix (Roche Diagnostics, Mannheim, Germany), or FastStart Universal Probe Master mix (Roche). Samples were analyzed using ABI 7300 QPCR system (Applied Biosystems) with fluorescence raw value correction to ROX reference values.

Target gene levels were normalized to reference gene 18S levels. Primer sequences for CYP2B10, CYP3A11 and 18S have been previously described (Lämsä et al., 2010). CYP2A6 levels were determined by priming with the following oligos: the forward primer was 5′-GGGCCAAGATGCCCTACATG and the reverse primer was 5′-AATGTCCTTAGGTGACTGGGA. TaqMan primers for CYP1A2 (Hs01070374_m1), CYP2B6 (Hs03044634_m1), CYP2C8 (Hs00258314_m1), CYP2C9 (Hs00426397_m1), CYP2C19 (Hs00426390_m1), CYP2D6 (Hs02576168_g1), CYP2E1 (Hs00559368_m1), and CYP3A4 (Hs00430021_m1) were purchased from Applied Biosystems (Foster City, CA, USA). Cycling parameters for each of the PCR reactions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.7. Determination of drug metabolizing enzyme activities by N-in-one assay

The N-in-one assay has been described previously (Tolonen et al., 2005, 2007). In brief, all 10 probe substrates were added as a mixture into the culture medium in a small volume of DMSO (final 0.5%, v/v) and incubated for 4 h. Substrates, their target CYPs and final concentrations in the cultures were: melatonin (CYP1A1/2, 40 μM), coumarin (CYP2A6, 20 μM), bupropion (CYP2B6, 10 μM), amodiaquine (CYP2C8, 20 μM), tolbutamide (CYP2C9, 40 μM), omeprazole (CYP2C19, 20 μM), dextromethorphan (CYP2D6, 2 μM), chlorzoxazone (CYP2E1, 60 μM), midazolam (CYP3A4, 4 μM) and testosterone (CYP3A4, 10 μM). Samples were collected then mixed with acetonitrile (1:1) containing fenasetin as an internal standard and centrifuged for 15 min at 10,000 × *g*. Supernatants were collected in clean tubes and stored at −20 °C until analyses. Before analysis, the incubations were thawed at room temperature, shaken, and centrifuged for 10 min at 10,000 × *g*. The supernatants were transferred to a Waters Total Recovery vial (Waters Corporation, Milford, MA, USA) for liquid chromatography–tandem mass spectrometry (LC/MS–MS) analysis, as described earlier in detail (Tolonen et al., 2005, 2007).

3. Results

3.1. Screening of human PXR and CAR activation by pesticides

We examined the ability of the test compounds to activate human nuclear receptors PXR and CAR by using reporter gene activity assays in HepG2 and C3A hepatoma cells, respectively. Functionality of the assays was first confirmed by positive control agonists. Rifampicin, a positive control for hPXR, enhanced luciferase activity 3.3-fold compared to DMSO-treated cells. Analogously, TMPP and FL81, positive control compounds for hCAR, enhanced luciferase activity 2.7- and 5.4-fold, respectively, compared to DMSO-treated cells.

Several pesticides were found to efficiently activate hPXR activity. Out of the 24 compounds tested, 14 increased luciferase activity at least 2-fold by either concentrations tested. The highest induction (4.0-fold over the control) was detected with 50 μM isoproturon. On the other hand, 10 μM isoproturon did not reach the 2-fold cut-off value. Alpha-cypermethrin activated hPXR to the same degree as rifampicin already at 10 μM concentration. Pyrethroids all consistently activated hPXR at least 2-fold already at 10 μM concentration while other types of pesticides were generally less efficient activators (Fig. 1).

Also hCAR was activated by a number of pesticides. Fifteen out of the 24 pesticides were able to increase luciferase activity 2-fold or more by at least by one of the concentrations tested. The highest induction was observed with 10 μM cypermethrin (5.6-fold), and benfuracarb (about 5-fold), irrespective of the dose (Fig. 1). While hPXR was predominantly induced by pyrethroids, hCAR was, in addition to pyrethroids, well activated by OPs and several carbamates.

3.2. Induction of CYP mRNA expression by pesticides in HepaRG cells

To assess the functional consequences of PXR and CAR activation, we next measured CYP mRNA induction by the same series of pesticides. We used the HepaRG cell line that, unlike most hepatoma cell lines, contains a relatively well preserved metabolic competence and expresses several key CYP enzymes (Anthérieu et al., 2010; Turpeinen et al., 2009). Furthermore, induction mediated by CAR and PXR is preserved in this cell line (Kanebratt and Andersson, 2008). The possible cytotoxicity of the studied pesticides was determined with the release of lactate dehydrogenase into the medium. However, negligible cytotoxicity was observed with the concentrations used (Suppl. Table 2).

Several model inducers were used to demonstrate inducibility of the different CYP forms. The cells were treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (1 and 10 nM; aryl hydrocarbon receptor (AhR) agonist), phenobarbital (PB) (0.5 and 1.5 mM; CAR activator), rifampicin (RIF) (10 and 50 μM; PXR agonist), dexamethasone (DEX) (50 and 250 μM; PXR agonist, but affect also other receptors such as glucocorticoid receptor), or DMSO (control cells) for 24 h after which the cells were collected, mRNA extracted and mRNA expression measured with RT-qPCR. The following panel of major inducible CYP mRNAs was studied: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, 2C19 and CYP3A4.

CYP mRNAs were induced by the model inducers as expected. CYP3A4 was induced 20-fold by RIF and to a lesser extent by PB and DEX. In contrast, CYP2B6 was induced 6.5-fold by 0.5 mM PB while RIF and DEX had only a modest effect. Regulation of CYP2A6 resembled that of CYP2B6. CYP1A2 was induced strongly, up to 239-fold, by TCDD and modestly also by 0.5 mM PB. Interestingly, in all cases 0.5 mM concentration of PB was more efficient inducer than 1.5 mM (Fig. 2). CYP2Cs were rather poorly induced by the model inducers. PB and/or RIF induced different CYP2 C mRNAs about 2-fold (Suppl. Fig. 1).

The human PXR activation assays suggested that pyrethroids are efficient activators of this nuclear receptor. In agreement with this, CYP3A4 mRNA was strongly induced by all tested compounds in this group. The highest level of CYP3A4 induction, 35-fold, was detected with 50 μM cypermethrin. Cypermethrin was actually a more powerful CYP3A4 inducer than the classical hPXR ligand RIF. Interestingly, all tested pesticides induced CYP3A4 mRNA 2-fold or more at least with the higher concentration used (Fig. 2).

The general induction pattern of CYP2B6 and CYP2A6 was distinct from CYP3A4. Pyrethroids, OPs, and carbamates were all fairly equal inducers of CYP2B6. This may reflect the fact that PXR was activated predominantly by pyrethroids, while CAR was activated more equally by all three classes of compounds. CYP2A6 mRNA induction profile was quite similar to CYP2B6, however, carbamates were poor inducers of CYP2A6 (Fig. 2).

Pyrethroids and carbamates had rather small effects on CYP1A2 expression. In contrast, all OP compounds were good inducers of this CYP form. Urea compounds isoproturon and diuron, and the triazine derivative atrazine were wide spectrum inducers and upregulated all CYP1A2, CYP2A6, CYP2B6 and CYP3A4 (Fig. 2). CYP2C8, CYP2C9 and CYP2C19 were barely inducible by the tested compounds. However, pyrethroid compounds slightly induced CYP2C subfamily members (Suppl. Fig. 1), and the highest induction of CYP2Cs was detected with fenvalerate.

3.3. Modulation of HepaRG CYP activity by pesticides

Enzymatic activity of different CYP enzymes was determined from the same samples as mRNAs by using the cocktail approach. Again, the effect of model inducers was first determined. CYP3A4-mediated activities testosterone 6β-hydroxylation, midazolam 1-hydroxylation, omeprazole 3-hydroxylation and omeprazole sulfoxidation were all similarly induced by PB, RIF and DEX (Fig. 3 and Suppl. Fig. 2). On the other hand, coumarin 7-hydroxylation (CYP2A6) and bupropion hydroxylation (CYP2B6) were only induced by PB (Fig. 3). Interestingly, in contrast with mRNA, CYP activities were induced dose-dependently by PB. Melatonin 6-hydroxylation was strongly induced by TCDD in agreement with the major involvement of the CYP1A2 enzyme. However, even RIF increased melatonin 6-hydroxylation activity about 6-fold

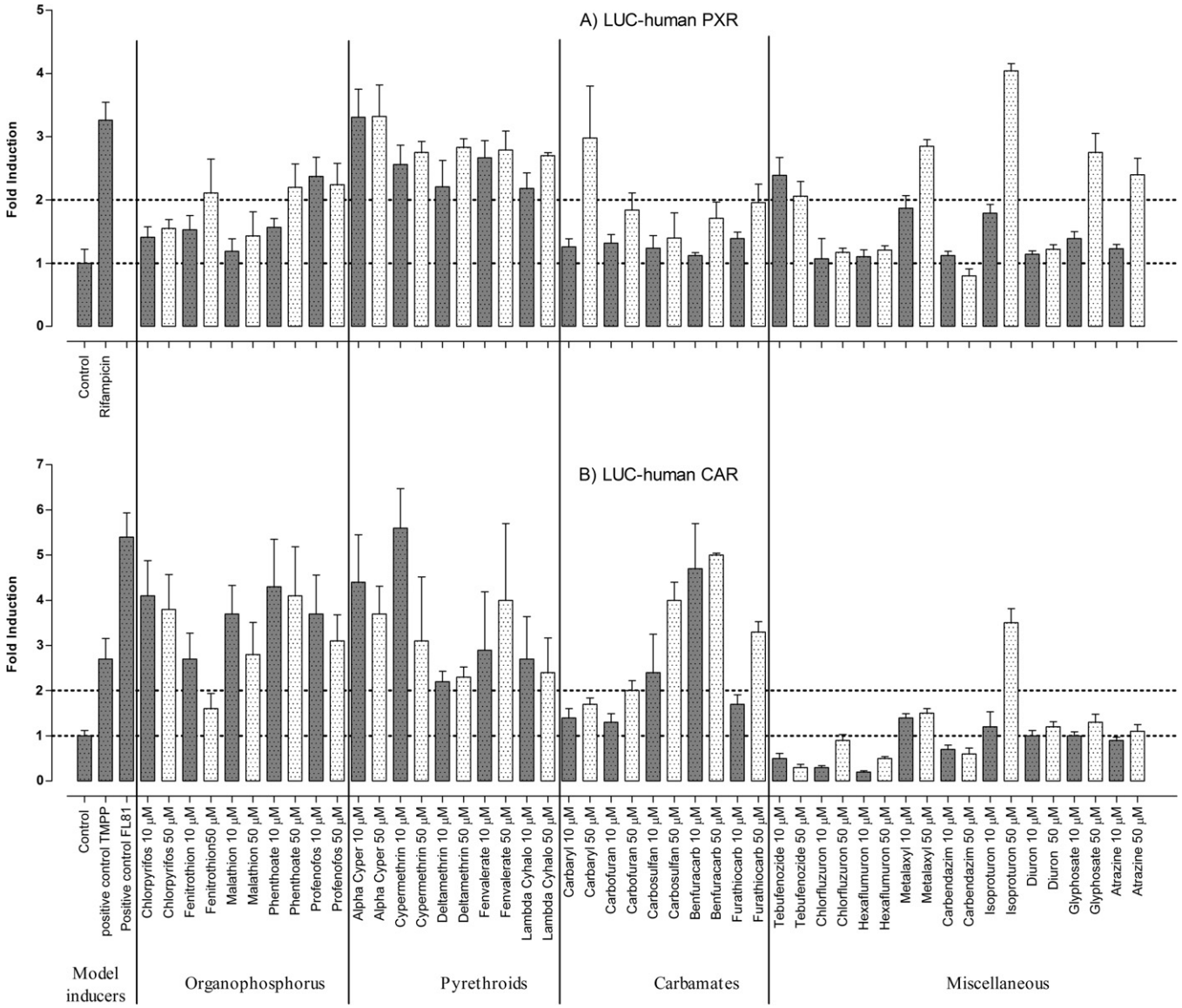

Fig. 1. Activation of human: (A) PXR and (B) CAR by model inducers and pesticides. Nuclear receptor activation was measured by luciferase reporter gene activity as described in Section 2. The results are expressed as mean ± SD of at least three independent experiments.

suggesting that this catalytic activity is not totally specific for CYP1A2 (Fig. 3).

Several CYP3A4-associated catalytic activities were highly correlated ($r^2 > 0.9$) (Fig. 3 and Suppl. Fig. 2). CYP3A4-mediated activities were induced to a lesser extent than the corresponding mRNA levels. Interestingly, this discrepancy was more pronounced for the pesticides than the model inducers. For example, RIF induced CYP3A4 mRNA and testosterone 6β-hydroxylation 20- and 10-fold, respectively, while 50 μM cypermethrin induced CYP3A4 mRNA 35-fold, but testosterone 6β-hydroxylation only 3.4-fold. However, the qualitative changes in CYP3A4 activity and mRNA expression generally agreed (Figs. 2 and 3).

The CYP2B6-mediated activity was induced to a lesser extent than the CYP2B6 mRNA by the pesticides. Particularly, OP compounds induced CYP2B6 mRNA 3–4-fold, but actually inhibited CYP2B6-mediated bupropion hydroxylation (Figs. 2 and 3). Curiously, glyphosate (10 and 50 μM) appeared to have a more powerful effect on CYP2B6 activity (5–9-fold) than mRNA expression (5–2-fold). Treatment of HepaRG with pesticides did not tend to increase CYP2A6-catalyzed coumarin 7-hydroxylation (Fig. 3). It has to be stressed here that 7-hydroxycoumarin peaks in the LC–MS chromatogram were very poor, probably because of low CYP2A6 activity in the cell line (Turpeinen et al., 2009). Effect of pesticides on the melatonin 6-hydroxylase activity correlated poorly with the effect on CYP1A2 mRNA ($r^2 = 0.13$).

3.4. Species differences of CYP induction

It is well established that different chemicals activate nuclear receptors and induce CYP genes in a species-dependent manner (Song et al., 2005; Lin, 2006). We tested whether mouse CYP2B10 and CYP3A11 mRNAs are induced by the test compounds in cultured mouse primary hepatocytes. CYP3A11 was induced by the pyrethroids, tebufenozide and metalaxyl in a similar fashion to CYP3A4 in HepaRG cells (Fig. 4). In fact, CYP3A4 and CYP3A11 induction responses correlated rather well ($r^2 = 0.57$) (Suppl. Fig. 3). CYP2B10 was induced quite poorly by the pesticides (Fig. 4).

Since human and mouse PXR activation by pesticides has recently been extensively characterized and compared (Kojima

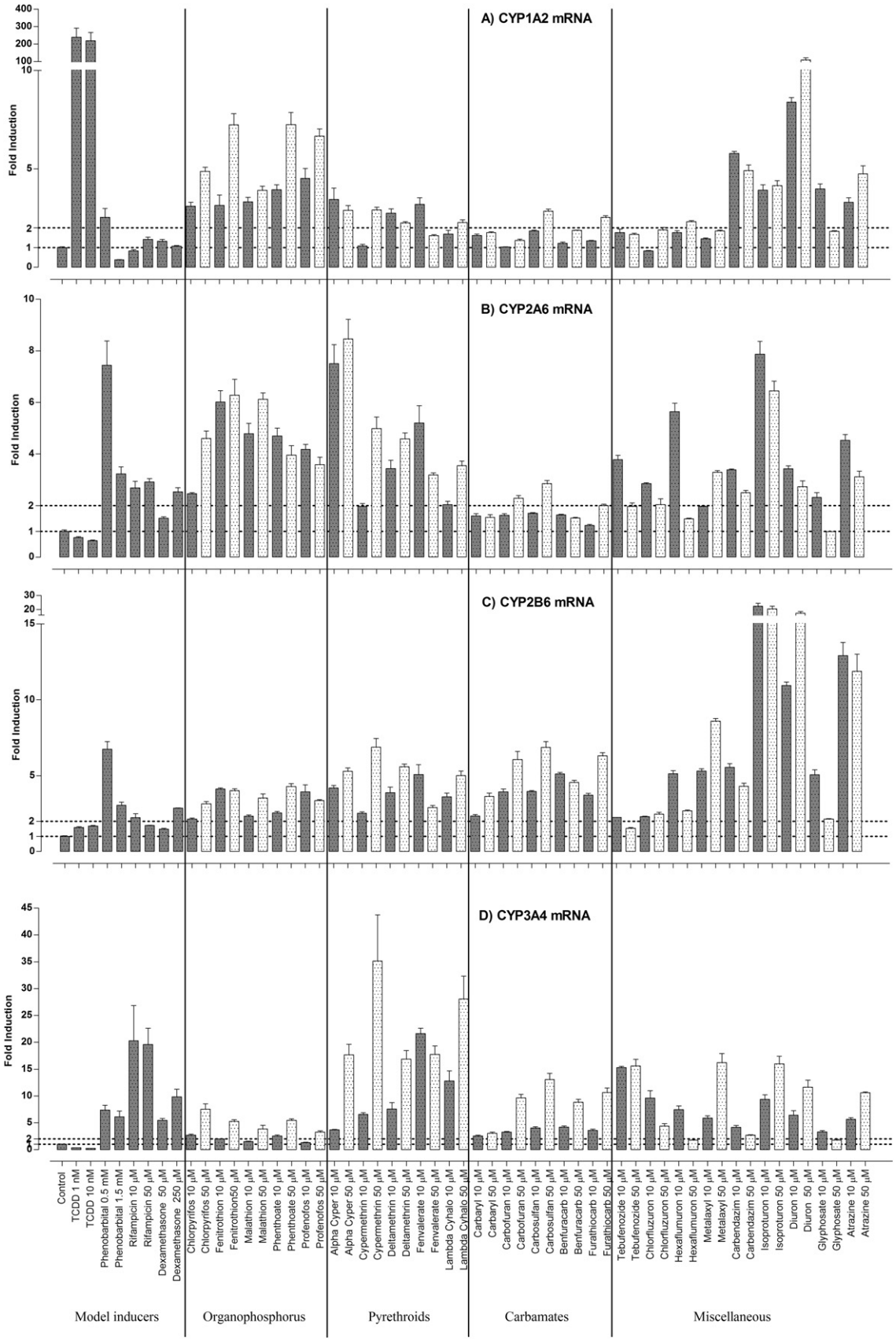

Fig. 2. CYP mRNA levels in human HepaRG hepatoma cell line after 24 h exposure to tested compounds. Total RNAs were isolated and subjected to RT-PCR analysis for the expression of (A) CYP1A2, (B) CYP2A6, (C) CYP2B6 or (D) CYP3A4 mRNAs. The mRNA levels are expressed as relative to the respective control cell level (normalized as 1) and the data are expressed as mean ± SD of six replicates.

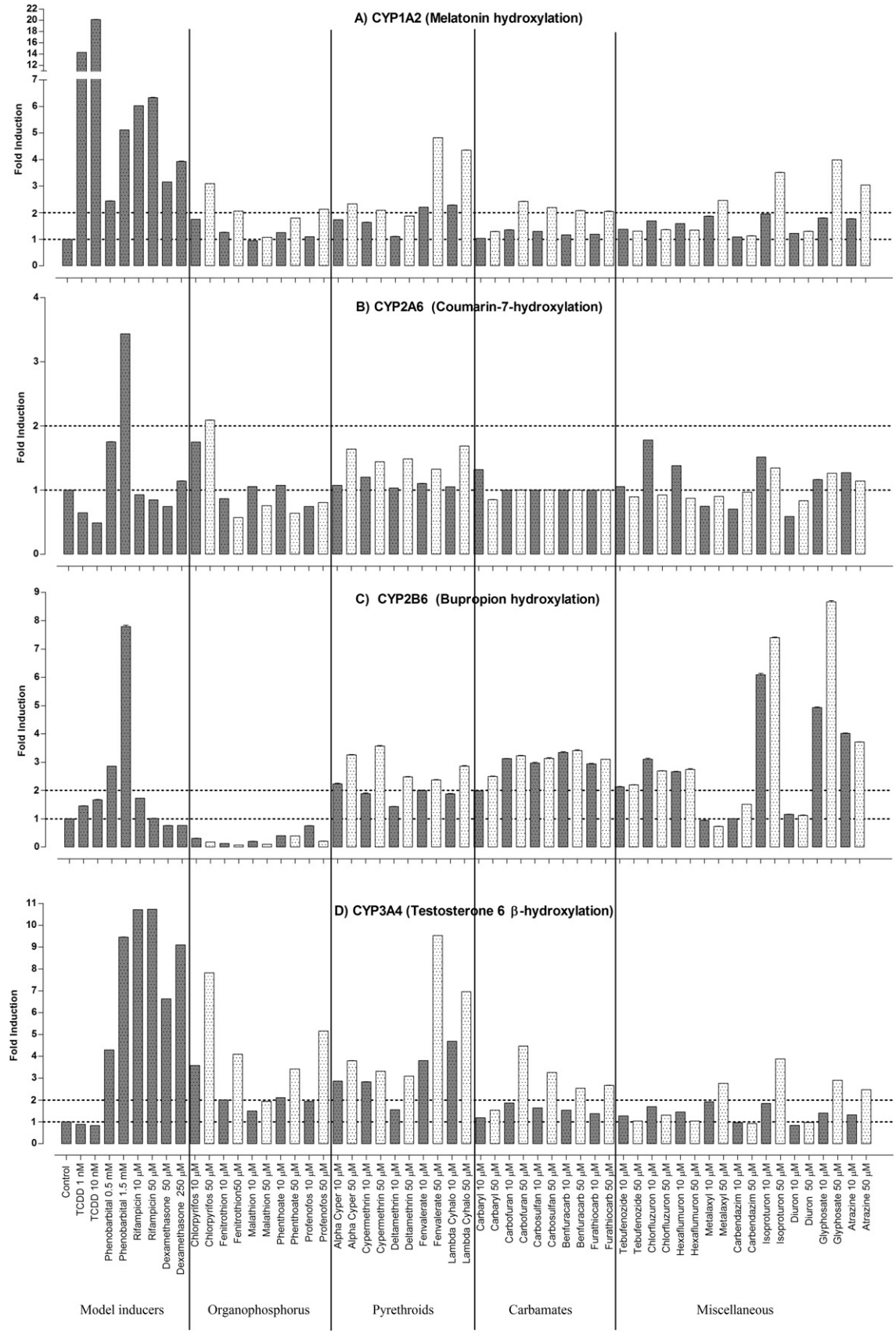

Fig. 3. CYP enzyme activities: (A) CYP1A2, (B) CYP2A6, (C) CYP2B6, (D) CYP3A4 measured by N-in-one assay. All probe substrates were added as a mixture into the culture medium in a small volume of DMSO (final 0.5%, v/v) and incubated for 4 h. Medium were collected and analyzed by liquid chromatography–tandem mass spectrometry (LC/MS–MS). The enzyme activity levels are expressed as relative to the respective control cell level (normalized as 1) and the data are expressed as mean ± SD of three replicates.

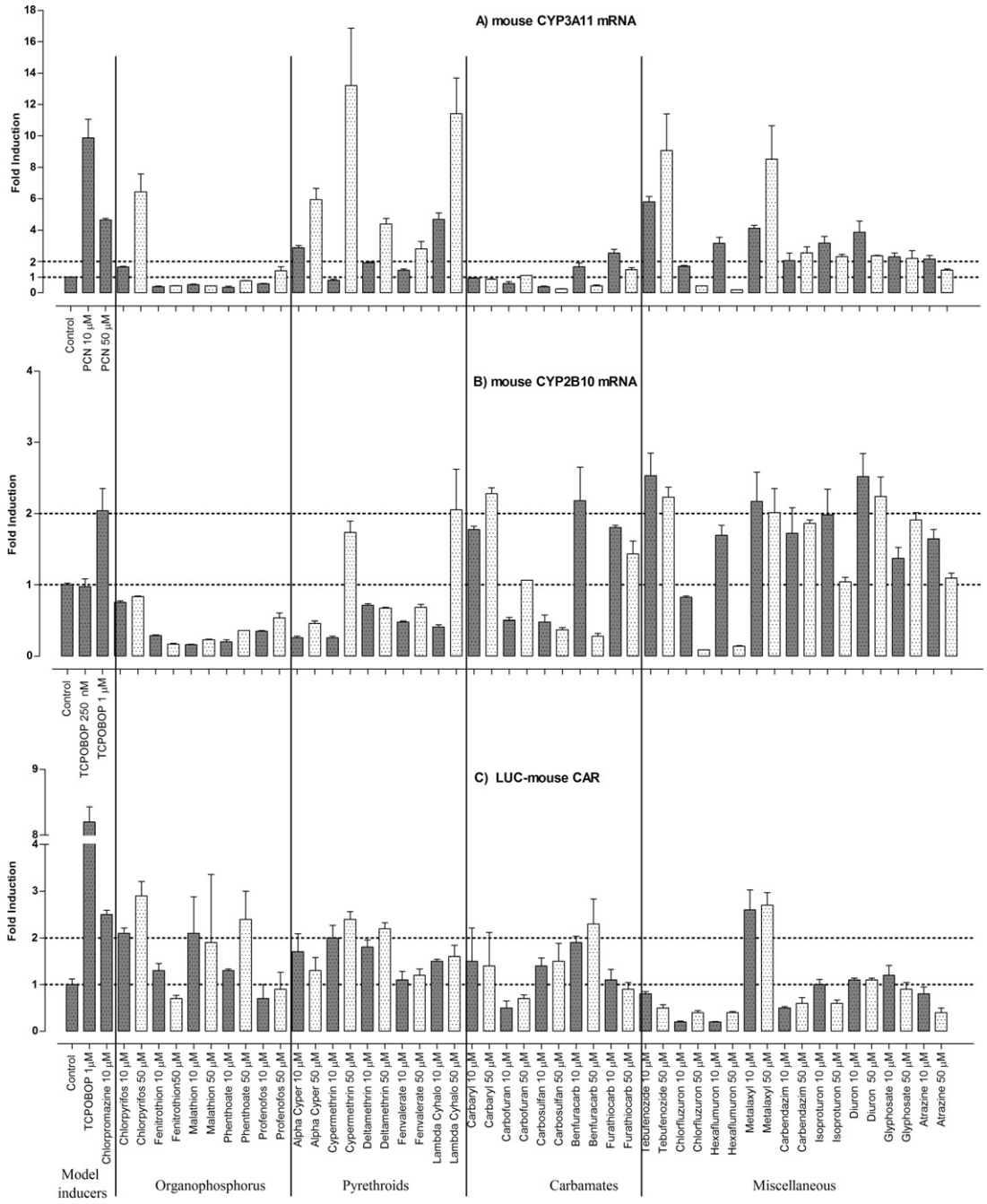

Fig. 4. (A) CYP3A11 and (B) CYP2B10 mRNA levels in mouse primary hepatocytes treated with model inducers and pesticides for 24 h. Total RNAs were isolated and subjected to RT-PCR analysis. The mRNA levels are expressed as relative to the respective control cell level (normalized as 1) and the data are expressed as mean ± SD of six replicates. (C) Activation of mouse CAR by model inducers and pesticides. Nuclear receptor activation was measured by luciferase reporter gene activity as described in Section 2. The results are expressed as mean ± SD of at least three independent experiments.

et al., 2011) we focused on the species-specific effects of pesticides on CAR activation. In the luciferase assays, TCPOBOP and chlorpromazine, established activators of mouse CAR (Tzameli et al., 2000; Wei et al., 2002), were able to induce luciferase activity 8.2 and 2.5-fold, respectively. Pesticides clearly had a less effect on mouse CAR than human CAR. None of the pesticides were able to activate mouse CAR over 3-fold. The highest activation (2.5-fold) was achieved by 50 μM chlorpyrifos, phenthoate, cypermethrin, benfuracarb and metalaxyl (Fig. 4). Poor activation of mouse CAR by the studied pesticides is in agreement with the low induction potential of CYP2B10.

4. Discussion

Toxicity of pesticides may be modulated by induction of drug-metabolizing CYP enzymes. In the current study, we have compared effects of 24 pesticides on activation of nuclear receptors PXR and CAR, and on induction of CYP mRNAs and catalytic activities in a metabolically competent cell model. Based on the actually measured chlorpyrifos plasma concentrations in human volunteers and pharmacokinetic models of the environmental exposure in U.S. adults, children and agricultural workers and the available data on human exposure (Nolan et al., 1984; Krieger and Dinoff, 2000;

Adgate et al., 2001; Rigas et al., 2001; Aprea et al., 2004) it has been suggested that, in vitro concentrations around 10–50 μM, which were used in our study, are the most appropriate concentrations to mimic in vivo conditions of exposure to OPs pesticides such as chlorpyrifos, azinphos-methyl, malathion and dimethoate (Buratti et al., 2003, 2005, 2002; Buratti and Testai, 2007). Although this may not be true for all types of pesticides it was used as the starting point for our study.

Both human PXR and CAR were found to be activated by a large number of pesticides. While PXR activation by pesticides has been recently explored in several studies (Yang et al., 2009; Kojima et al., 2011; Coumoul et al., 2002), less is known about the role of CAR. Our study indicates that CAR is an equally important target of pesticides as PXR is. While pyrethroids efficiently activated both nuclear receptors, OP and carbamate compounds were generally better activators of CAR. This was also reflected in CYP mRNA induction. While CYP3A4 mRNA was induced strongly by pyrethroids, CYP2B6 was equally induced by all three compound classes.

Pyrethroids moderately induced CYP2A6 and CYP2B6 mRNAs, and strongly upregulated CYP3A4 mRNA in HepaRG cells. These results are in agreement with several previous reports. Yang et al. (2009) reported that lambda-cyhalothrin, deltamethrin and fenvalerate induced CYP3A4 in multiple cell lines, including HepG2, Hop92 and LS180. Fenvalerate and cypermethrin induced CYP3A4 and CYP2B6 (Lemaire et al., 2004) and permethrin induced CYP2B6 and CYP2A6 in primary cultures of human hepatocytes (Hodgson and Rose, 2007). The induction potency of pyrethroid compounds may be due to the presence of cyano moiety, which facilitates the interaction with PXR, and the phenoxybenzyl group, which along with the cyano moiety forms a bulky and lipophilic platform (Yang et al., 2009).

Our study demonstrates that OPs induce CYP1A2, CYP2A6, CYP2B6 and CYP3A4 mRNA in HepaRG cells. Several studies have been published on the chlorpyrifos-CYPs interactions, while few data are available on other OPs. Chlorpyrifos has been reported to induce CYP1A1, 1A2, 2A6, 2B6 and 3A4 in primary cultures of human hepatocytes (Lemaire et al., 2004; Hodgson and Rose, 2007) and CYP1A1 mRNA in HepG2 (Rudzok et al., 2009) through binding to the aryl hydrocarbon receptor (AhR) (Takeuchi et al., 2008; Long et al., 2003). We did not investigate AhR-mediated regulation in the current study, but the efficient induction of CYP1A2 suggests activation of this ligand-dependent transcription factor by the OPs. However, human CAR was also activated by all OPs studied, which most probably explains the inducibility of CYP2A6 and CYP2B6. CYP3A4 mRNA was induced only by high concentrations of OPs.

Little is known on the inducibility of CYPs by carbamate compounds. A moderate CYP1A1 induction, as detected by ethoxyresorufin O-deethylase activity and by Northern blots, was observed with carbaryl in rat and human hepatocyte cultures (de Sousa et al., 1997). We found that carbamate compounds increase expression of CYP2B6 and CYP3A4 mRNAs in the HepaRG cell line. The nuclear receptor activation assays suggest that this is mediated predominantly by CAR. Interestingly, CYP2A6, which participates in the metabolism of carbaryl (Tang et al., 2002) and carbosulfan activation (Abass et al., 2010) was poorly induced by carbamates.

Isoproturon, diuron and atrazine induced numerous CYP mRNAs as shown by strongly increased CYP1A2, CYP2A6, CYP2B6 and CYP3A4 mRNA levels in HepaRG. Induction of CYP1A2 by diuron is in agreement with previous studies showing induction of both CYP1A1 and 1A2 mRNAs and activation of AhR by diuron (Takeuchi et al., 2008; Rudzok et al., 2009). Of these three compounds, only isoproturon significantly activated CAR indicating that some pesticides induce CYP2A6 and CYP2B6 via CAR-independent mechanisms, possibly through PXR.

Many pesticides are metabolized by CYP enzymes and enzyme induction can directly affect the detoxification and activation processes. OPs (Buratti et al., 2003, 2005, 2002; Mutch and Williams, 2006; Abass et al., 2007a; Buratti and Testai, 2007; Mutch et al., 2003; Sams et al., 2000) and carbamate insecticides (Usmani et al., 2004; Abass et al., 2010; Tang et al., 2002) are mainly activated by CYP3A4. Based on our current results, OP and carbamate compounds induce CYP3A4 mRNA and catalytic activity which may enhance the neurotoxicity of these compounds via the enhanced oxidative biotransformation. CYP2B6 mediates chlorpyrifos and malathion activation (Buratti et al., 2005; Mutch and Williams, 2006; Foxenberg et al., 2007; Sams et al., 2000; Tang et al., 2001), carbosulfan sulfoxidation (Abass et al., 2010) as well as profenofos detoxification and activation (Abass et al., 2007a). All these compounds also induced CYP2B6 mRNA expression. We have previously shown the importance of CYP2B6 and CYP3A4 in metalaxyl detoxification in human hepatic microsomes in vitro (Abass et al., 2007b). Metalaxyl induced CYP2B6 and CYP3A4 mRNA strongly in HepaRG indicating that metalaxyl can induce its own metabolism.

It is notable that OPs induced the CYP mRNAs, however, the catalytic activities were not raised in a similar fashion or they were even inhibited. All the tested OPs are efficient inhibitors of CYP1A1/2, CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP3A4 activities (Abass et al., 2009b). CYP-mediated biotransformation is the first step and critical event in the activation of OPs. The reactive sulphur released during the bioactivation of OPs via CYP-mediated desulfuration to their phosphate oxon metabolites binds irreversibly to the heme iron of CYP, inhibiting its activity through the mechanism of suicidal inhibition (Neal, 1980; Neal and Halpert, 1982; Butler and Murray, 1997). Thus, the CYP inhibition can be interpreted as a sign of bioactivation. As a consequence, the enzyme activity is a poor indicator of CYP gene regulation by OP pesticides.

Measurement of nuclear receptor activation appears to be a fairly good qualitative indicator of CYP induction potential by the pesticides, and compounds predicted to activate CAR and/or PXR did induce the expected CYP mRNAs. However, the quantitative estimation of CYP mRNA induction from the induction of nuclear receptors is usually not accurate. Furthermore, some compounds may induce CYPs through mechanisms independent of CAR and/or PXR. For example, chlorfluzuron and hexaflumuron induced CYP2A6, CYP2B6 and CYP3A4 but did not activate either CAR or PXR. Indeed, regulation of CYPs is a complex phenomenon and include other mechanisms besides PXR and CAR (Honkakoski and Negishi, 2000; Murray et al., 2010; Anderson et al., 2011). Therefore, the nuclear receptor activation assays should not be used as the sole method to identify CYP inducers.

Nuclear receptors CAR and PXR are known to display larger interspecies differences in ligand binding (Kretschmer and Baldwin, 2005). A recent study by Kojima et al. (2011) reported relatively good agreement of human and mouse PXR activation by pesticides. Of the 200 pesticides tested, 111 displayed hPXR and/or mPXR activation, of which 88 were common PXR activators. In agreement with this, we observed a positive correlation between the CYP3A4 induction in HepaRG and the CYP3A11 induction in mouse primary hepatocytes by the studied pesticides. In contrast, based on our comparison of the mouse and human CAR activation assays and also CYP2B mRNA induction, the mouse CAR seems to be much less responsive to pesticides than the human receptor. The observed differences emphasize the importance of using human-based cellular screening models in risk assessment.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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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.tox.2012.01.010.

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