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DMD #13144

Title page

Title:

Assessment of human pregnane X receptor-involvement in pesticide-mediated activation of CYP3A4 gene

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Running title page

a) Running title: RNAi inhibition of hPXR-mediated gene activation

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The number of words in the *Discussion*: 649

d) Abbreviations used in this paper: ANOVA, analysis of variance; CAT, 2-chloro-4,6-bis(ethylamino)-1,3,5-triazin; CAR, constitutive androstane receptor; CTZ, clotrimazole; P450, cytochrome P450; DMSO, dimethylsulfoxide; EPN, *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hPXR, human PXR; VDR, vitamin D receptor; MEP, *O,O*-diethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate; MOI, multiplicity of infection; mPXR, mouse PXR; PCR, polymerase chain reaction; PXR, pregnane X receptor; RT-PCR, reverse transcription-polymerase chain reaction; RIF, rifampicin; siRNA, small interfering RNA; $TCID_{50}$, 50% titer culture infectious dose; TPN, tetrachloroisophthalonitrile; VD_3, 1α,25-dihydroxyvitamin D_3.

Abstract

Assessment of foreign chemical-inducibility on CYP3A4 is necessary to optimize drug therapies. The properties of chemicals such as pesticides, however, are not well investigated. In the present study, properties of various pesticides on human CYP3A4 induction have been tested using HepG2-derived cells stably expressing the *CYP3A4* promoter/enhancer (3-1-10 cells) and the human pregnane X receptor (hPXR)-small interfering RNA (siRNA) system. Among the examined pesticides, 13 pesticides were observed to activate the CYP3A4 gene. Surprisingly, pyributicarb was found to increase the *CYP3A4* reporter activity at 0.1 to 1 μM more strongly than typical CYP3A4 inducer rifampicin. Expression of hPXR-siRNA clearly diminished the pyributicarb-stimulated *CYP3A4* reporter activity in 3-1-10 cells and decreased the endogenous CYP3A4 mRNA levels in HepG2 cells. Pyributicarb caused enhancement of *CYP3A4*-derived reporter activity in mouse livers introduced with hPXR by adenovirus. These results indicate pyributicarb as a potent activator of CYP3A4 gene, suggesting the existence of pesticides leading to CYP3A4 induction in our environment.

Introduction

Activities of drug-metabolizing enzymes often show large inter-individual differences, which leads to inter-individual variation in the efficacy of therapeutic drugs and/or severities of their adverse effects. The variation results not only from genomic backgrounds but also physiological and environmental factors such as nutrition and disease state and exposure to foreign chemicals including therapeutic drugs. Cytochrome P450 3A4 (CYP3A4) is an enzyme demonstrating large inter-individual differences (Wolbold et al., 2003). This form is a major P450 contained in human livers (Gonzalez, 1992) and involved in metabolisms of about a half of clinically used drugs (Li et al., 1995). The exact mechanism causing the differences in the expression levels of CYP3A4 in human livers remains unclear, although genetic variation is considered as a reason for the difference of CYP3A4 expression. Endogenous and exogenous chemical-mediated transactivation of CYP3A4 gene may however be one of the underlying mechanisms.

To develop a screening system which detects CYP3A4 gene activators with high sensitivity, we established the HepG2-derived cell lines stably expressing the CYP3A4-luciferase reporter gene (bases -362 to +11 and -7836 to -7008) (Norachartttiyapot et al., 2006). The reporter gene contains pregnane X receptor (PXR) binding sites as reported previously (Hashimoto et al., 1993; Barwick et al., 1996; Goodwin et al., 1999). PXR is widely known as a major transcription factor mediating CYP3A4 induction (Moore and Kliewer, 2000), and interacts with a wide variety of therapeutic drugs including rifampicin (RIF) and clotrimazole (CTZ) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). Among the established

cell lines, 3-1-10 showed the highest response to RIF (Noracharttiyapot et al., 2006). Thus, this cell line was chosen to assess chemical-inducibility of the CYP3A4 gene.

In addition to PXR, other receptors such as constitutive androstane receptor (CAR) (Goodwin et al., 2002) and vitamin D receptor (VDR) (Schmiedlin-Ren et al., 2001; Thummel et al., 2001) have also been reported to activate the transcription of CYP3A4 gene. Understanding whether a chemical can activate nuclear receptors is an inevitable step to predict the inducibility of CYP3A4. However, the involvement of PXR in the chemical-mediated CYP3A4 induction is not precisely determined using the PXR binding sites because CAR and VDR are capable of interacting with the sites. Recently, introduction of specific short nucleotides (small interfering RNA, siRNA) in cells has been shown to specifically knock down the target gene expression (Elbashir et al., 2001). Human PXR (hPXR)-siRNA is, however, scarcely used to understand the mechanisms of the endogenous and exogenous chemical-mediated activation of the CYP3A4 gene. The development of an effective hPXR-siRNA probe may stimulate clear identification of PXR involvement in chemical-mediated CYP3A4 induction.

Pesticides are among those xenobiotic candidates that may cause inter-individual variation of CYP3A4 expression, since they are widely used for animals, insects and plant, and released into the environment. They can be taken into human bodies via food, water and air. There is a possibility of the existence of a chemical modulating the CYP3A4 expression through hPXR activation. Although pesticides have been reported to activate PXR or CAR (Wyde et al., 2003;

Jacobs et al., 2005; Lemaire et al., 2006), pesticide-mediated activations of CYP3A4 gene are not well assessed.

In this study, we have developed an adenovirus vector expressing hPXR-siRNA that is able to specifically knock down hPXR expression. A combination of the 3-1-10 cell line and the hPXR-siRNA system enabled us to efficiently identify a potential hPXR activator causing CYP3A4 induction. These results suggest the possibility that some pesticides contaminated in the environment potentially induce CYP3A4 expression through hPXR.

Materials and Methods

Materials

Protein Assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). Restriction enzymes were purchased from New England BioLabs (Beverly, MA). RIF, CTZ and VD_3 were purchased from Sigma-Aldrich (St. Louis, MO). All pesticides were purchased from Wako Pure Chemical industries, Ltd (Osaka, Japan).

Animal treatment and Cell culture

Male ICR mice (5 weeks old) were purchased from Charles River Laboratories Japan, Inc (Yokohama, Japan) and fed standard rodent chow (CE-2; CLEA, Tokyo, Japan) and water *ad libitum*. After 18-hour fasting, mice were injected intravenously with adenovirus (4.0×10^9 $TCID_{50}$/mouse). Three days after the infection, vehicle (0.5% methyl cellulose/saline) or pyributicarb (100 mg/kg/day) was administered orally for 2 consecutive days. Animals were killed 20 hours after the last dose. Cell culture and adenovirus infection were carried out as described previously (Furukawa et al., 2002). Methylthiazole tetrazolium incorporation assay was carried out according to the method described by Dr. Jaiswal (Jaiswal et al., 2004).

Construction of recombinant adenovirus

AdhPXR-siRNA: Human U6 snRNA gene promoter was used for the expression of siRNA. The U6 snRNA promoter was amplified by PCR with primers, 5´-CGCTCGAGCCGACGCCGCCATCTC-3´ and

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5´-GCGTCTAGAGTTAACAAGGCTTTTCTCCAAGGG-3´. The PCR product was digested with *Xho*I and *Xba*I and cloned into the same restriction sites of the promoterless vector pShuttle (pShuttle-U6). The DNA encoding human PXR-specific siRNA was amplified by PCR with primers, 5´-GCGGTCGACGAGCTGTGTCAACTGAGATTCTTCAAGAGA-3´ and 5´-GCGAGATCTAAAAAGAGCTGTGTCAACTGAGATTCTCTCTTGAAGA-3´, and the PCR product was digested with *Sal*I and *Bgl*II, and ligated into the same restriction sites of pShuttle-U6 (pShuttle-U6-hPXR-siRNA). To obtain an adenovirus expressing hPXR-siRNA, the homologous recombination was utilized in BJ5183 cells transfected with pShuttle-U6-hPXR-siRNA linearized with *Pme*I and adenoviral backbone plasmid pAdEasy-1 (Quantum Biotechnologies, Quebec, Canada). The resultant DNA was linearized with *Pac*I, and HEK293 cells were transfected with the DNA using CellPhect Transfection Kit (GE Healthcare, Piscataway, NJ). Target sequences for hPXR-siRNAs were selected manually at random basis. The adenovirus expressing hPXR-siRNA used in this study showed the highest efficiency among four siRNAs tested on the knock-down of hPXR mRNA levels (data not shown).

AdCYP3A4-362-7.7k: The CYP3A4 enhancer region (from -7836 to -7208) was amplified by PCR with oligonucleotides 5´-CGACGCGTCTAGAGAGATGGTTCATTCC-3´ and 5´-GCAGATGTAATGATCTCGTCAACAGG-3´. The PCR products were digested with *Mlu*I and *Bgl*II and ligated into the same restriction sites of the pGLCYP3A4-362 (Furukawa et al., 2002). AdCYP3A4-362-7.7k was then obtained using the method reported previously

(Furukawa et al., 2002).

AdhPXR was reported previously (Norachartttiyapot et al., 2006). AdCont (AxCALacZ), which expresses β-galactosidase, was provided by Dr. Izumi Saito (Tokyo University). We demonstrated that this adenovirus did not affect either hPXR mRNA levels or the CYP3A4 induction by RIF in HepG2 cells as an adenovirus expressing non-targeting siRNA, mouse PXR-targeting siRNA which does not match to any human mRNAs (data not shown). Thus, we used AdCont as a control for both overexpression and RNA knockdown experiments. The titer of adenoviruses, 50% titer culture infectious dose ($TCID_{50}$), was determined in HEK293 cells. The value of $TCID_{50}$ was reported to be almost equivalent to that of plaque-forming unit (Kanegae et al., 1994). Multiplicity of infection (MOI) was calculated by dividing $TCID_{50}$ with the number of cells.

Detection of mRNAs

Total RNAs were extracted from HepG2 cells using the acid guanidine thiocyanate-phenol-chloroform method. The cDNA was reverse-transcribed from total RNA (30 μg) with Ready-To-Go™ (GE Healthcare). PXR, CYP3A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined by conventional or real-time PCR with Taq DNA polymerase (ABgene, Epsom, UK) as follow: After initial denaturation at 94˚C for 5 min, DNAs were amplified for 30 (PXR and GAPDH) or 39 cycles at 94˚C for 30 sec, at 57 for 15 sec (PXR and GAPDH) or 63˚C for 5 sec (CYP3A4),

at 72˚C for 30 sec with final extension period at 72˚C for 7 min. CYP3A4 and GAPDH mRNA levels were also determined by real-time PCR using Platinum® qPCR (Invitrogen, Carlsbad, CA) with ABI PRISM® 7000 (Applied Biosystems, CA) following the manufacturers' instructions. The sequences of primers and probes are shown in Table 1.

Luciferase assay

In vitro assay: The luciferase assay with cells was performed as reported previously (Furukawa et al., 2002). *In vivo* assay: Mouse livers were homogenized in a double volume of 25 mM Tris-HCl buffer (pH 7.4) and the homogenate was centrifuged at 9,000 x*g* for 20 min. The supernatant was centrifuged at 105,000 x*g* for 60 min. The resultant supernatant was used for the luciferase assay with Luciferase assay system (Promega, Madison, WI).

Preparation of PXR antibody

A portion of hPXR (amino acids 129 to 235) was expressed as a histidine-tagged protein with QIAexpressionist (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The expressed protein was purified with Ni-NTA column (Qiagen) under the denatured condition, then the histidine tag was removed with enterokinase. Rabbit was immunized with the recombinant protein, and anti-sera was affinity-purified with the same antigen immobilized to CNBr-activated Sepharose™-4B (GE Healthcare). The purified anti-sera detected both the antigen and hPXR protein expressed in COS-1 cells at expected molecular sizes. Apparent

molecular weight of the detected hPXR proteins in the HepG2 extract is 51.8 kDa (cf. Fig. 3B), which is similar to both that calculated from amino acids and that reported previously (Lehmann et al., 1998).

Immunoblot analysis

Nuclear extracts were prepared from 3-1-10 cells according to the method reported previously (Ogino et al., 1999), and separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was immunostained with the anti-PXR antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG, and signals were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium.

Statistical analysis

GraphPad Prism® version 4.00 (GraphPad Software, San Diego, CA) was used for all statistical analysis.

Results

Transcriptional activation of CYP3A4 reporter gene by pesticides

We investigated whether pesticides activate the *CYP3A4* transcription using the HepG2-derived cells stably expressing the CYP3A4 reporter gene (3-1-10 cells). The cells were treated with 0.3 to 30 μM of pesticides for 48 hours. Then reporter activities were determined (Fig. 1). Seventeen pesticides were tested in this study, which included 5 insecticides [chloropryfos, *O,O*-diethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate (MEP), etoxazole, *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) and isoxathion], 5 fungicides [tetrachloroisophthalonitrile (TPN), echlomezol, isoprothiolane, mepronil and flutolanil] and 7 herbicides [chloroneb, 2-Chloro-4,6-bis(ethylamino)-1,3,5-triazin (CAT), propyzamide, pyributicarb, ditiopyr, isofenphos and dymron]. RIF was used as a positive control. Among the insecticides EPN and isoxathion showed strong activation of the *CYP3A4* reporter (the maximum activation by EPN and isoxathion was 30.2- and 16.2-fold, respectively, both at 10 μM). For the fungicides, mepronil and flutolanil increased the activity (the maximum activation by mepronil and flutolanil was 14.6- and 20.5-fold, respectively, both at 30 μM). In herbicides, pyributicarb, ditiopyr, isofenphos and dymron were found to activate strongly the *CYP3A4* reporter (the maximum activation by pyributicarb, ditiopyr, isofenphos and dymron was 22.1-fold at 3 μM, 11.4-fold at 10 μM, 18.5-fold at 10 μM, and 12.2-fold at 10 μM, respectively). Surprisingly, the CYP3A4 reporter activities were higher with pyributicarb than with RIF at 0.3 and 1 μM (Fig. 1). Thus we compared the dose-dependent change of the activation of the CYP3A4 reporter gene by pyributicarb with those by typical hPXR activators

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(RIF and CTZ) in HepG2 cells (Fig. 2). Pyributicarb and RIF showed EC_{50} values of 0.21 and 2.2 μM, respectively, for CYP3A4 reporter activities in the transiently transfected HepG2 cells. Pyributicarb was a more potent activator of the *CYP3A4* reporter than RIF and CTZ.

Role of hPXR in the pesticide-mediated activation of the CYP3A4 promoter

To verify the mechanism that pesticides activate the CYP3A4 reporter gene, we have constructed a recombinant adenovirus expressing hPXR-siRNA. 3-1-10 cells were infected with AdhPXR-siRNA and treated with RIF, CTZ or VD_3 to confirm the specificity of this system. As shown in Fig. 3A, introduction of the adenovirus drastically diminished the RIF- and CTZ-mediated activation of the reporter gene but not that induced by VD3. Infection with AdhPXR-siRNA drastically decreased the amount of nuclear hPXR proteins (Fig. 3B). Effects of hPXR-siRNA expression on the endogenous CYP3A4 mRNA levels in HepG2 cells were also investigated (Fig. 3C). Introduction of hPXR-siRNA decreased hPXR mRNA levels. This treatment also inhibited strongly the RIF- stimulated, but not VD3-stimulated, increase of the endogenous CYP3A4 mRNA levels.

Using the hPXR-siRNA system, we found that the pyributicarb-, isoxathion- and EPN-mediated activation of the *CYP3A4* reporter was decreased by the introduction of AdhPXR-siRNA as in the case of RIF in 3-1-10 cells (Fig. 4A). Effects of the hPXR-siRNA expression on endogenous CYP3A4 mRNA levels in HepG2 cells were also investigated (Fig. 4B). Pyributicarb enhanced levels of CYP3A4 mRNA in HepG2 cells as RIF did. The

introduction of hPXR-siRNA attenuated the pesticide-mediated increase of the mRNA levels. Similar decreases were also observed with isoxathion and EPN.

We further investigated whether pyributicarb activated the *CYP3A4* promoter by *in vivo* reporter assay. Mice were infected with AdCYP3A4-362-7.7k and AdCont or AdhPXR, and then treated with pyributicarb. As shown in Fig. 5, mouse PXR activator pregnenolone 16α-carbonitrile (PCN) treatment increased *CYP3A4* reporter activity (53-fold). On the other hand, pyributicarb treatment did not increase reporter activity. Introduction of hPXR increased *CYP3A4* reporter activity (153-fold). Pyributicarb treatment increased *CYP3A4* reporter activity (623-fold) with introduction of hPXR, although it did not increase reporter activity without hPXR (Fig. 5).

Discussion

With the advantage of the 3-1-10 cell which stably expresses a CYP3A4 reporter gene, we have examined properties of seventeen functionally and structurally different pesticides for induction of CYP3A4 expression. Among them, 13 pesticides activated the CYP3A4 reporter gene (Fig. 1). EPN, isoxathion and pyributicarb also increased the levels of endogenous CYP3A4 mRNA in HepG2 cells (Fig. 4B). Pyributicarb was found to activate the *CYP3A4* reporter more strongly than RIF at submicromolar concentrations in both 3-1-10 and HepG2 cells (Figs. 1 and 2), indicating a possibility that this pesticide functions as a potent hPXR activator *in vivo*. At high concentrations, pyributicarb-induced activation was, however, diminished. This may result from its characteristics as a herbicide. Viability of pyributicarb cells, estimated by methylthiazole tetrazolium incorporation assay, was decreased to 37% that of vehicle-treated cells after 48-hour treatment with 30 μM of pyributicarb (data not shown). Nevertheless, utilization of a stable cell line 3-1-10 cells enabled us to identify herbicides that may cause CYP3A4 induction.

Chemical-induced activation of CYP3A4 gene is mainly mediated by PXR heterodimerized with retinoid X receptor through their binding to the *CYP3A4* 5´-flanking region (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). In addition to PXR, other nuclear receptors including CAR and VDR are also known to activate CYP3A4 gene. To determine whether hPXR is involved in the pesticide-mediated activation of CYP3A4 gene, we developed an AdhPXR-siRNA system and applied this to the reporter assays. Introduction of AdhPXR-siRNA drastically decreased the amount of nuclear hPXR proteins and resulted in the

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diminishment of the RIF- and CTZ-mediated activation but not that mediated by VD_3 (Fig. 3). When AdhPXR-siRNA was introduced into the 3-1-10 cells, the pyributicarb-, isoxathion- and EPN-mediated activation of the *CYP3A4* reporter was drastically attenuated (Fig. 4A). Furthermore, the introduction of AdhPXR-siRNA into HepG2 cells prevented the pesticide-induced increase of endogenous CYP3A4 mRNA levels (Fig. 4B). These results suggest that pyributicarb, isoxathion and EPN enhance the transactivation of the CYP3A4 gene through hPXR activation.

In the present study, we focused on the PXR-mediated transactivation of CYP3A4 gene, although a considerable role of CAR in the CYP3A induction has been reported. Some pesticides including methoxychlor and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane activate CAR and induce CYP3As (Nims et al., 1998; Blizard et al., 2001). Since our system has limited ability to detect CYP3A4 induction through human CAR because of little expression of CAR in HepG2 cells, experiments with human hepatocytes with AdhPXR-siRNA are currently underway in our laboratory to discern the role of PXR and CAR in the chemical-mediated induction of CYP3A4 including pesticides.

With the use of the adenovirus system, which is applicable to *in vivo* living organisms, we further investigated whether pyributicarb activated the CYP3A4 reporter gene in mice. Since there are differences of ligand specificity between hPXR and mouse PXR (mPXR) (Ostberg et al., 2002), we determined the reporter activity both in the absence and presence of hPXR. In the absence of exogenous hPXR, pyributicarb did not increase the *CYP3A4* reporter activity.

Introduction of hPXR itself increased *CYP3A4* reporter activity, and pyributicarb treatment further enhanced reporter activity (Fig. 5). In addition, a potent mPXR ligand, PCN, activated the *CYP3A4* reporter (Fig. 5), which excludes the possibility that the ligand-activated mPXR may not bind to the *CYP3A4* promoter. These results indicate that pyributicarb is a potent hPXR activator and a weak, if any, ligand of mPXR.

In conclusion, we have investigated the pesticide-inducibility of CYP3A4 gene expression using a HepG2-derived cell line expressing the CYP3A4 reporter gene and the AdhPXR-siRNA system, and identified plural hPXR activators among pesticides. Further studies, including the determination of pesticide exposure levels, are necessary to assess the *in vivo* significance of their human CYP3A4 induction. The screening system presented in this study is useful to understand the molecular mechanism of the xenobiotic-induced expression of CYP3A4 gene as well as to estimate the properties of chemicals on CYP3A4 induction.

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Footnote

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Legend for Figures

Figure 1 Activation of CYP3A4 reporter gene by pesticides in 3-1-10 cells.

3-1-10 cells (5.0 x 10^4 cells/well) in a 24-well plate were incubated with the vehicle (0.1% dimethylsulfoxide, DMSO), 0.3 to 10 μM RIF or 0.3 to 30 μM pesticides for 48 hours and luciferase activities were measured. Reporter activities are expressed as fold to that in the vehicle-treated cells. Data represent the mean ± SD (n = 4). *, $P < 0.05$; **, $P < 0.01$; significant difference from the vehicle-treated cells based on one-way ANOVA followed by a Dunnett's *post hoc* test.

Figure 2 Dose-dependent activation of CYP3A4 reporter gene by pesticides.

HepG2 cells (2.0 x 10^4 cells/well) in a 24-well plate were infected with AdCYP3A4-362-7.7k (MOI of 50) and were treated with vehicle (0.1% DMSO), 0.003 to 3 μM of pyributicarb, RIF or CTZ for 48 hours, then luciferase activities were determined. The activities are expressed as fold to those in the vehicle-treated cells. Data represent the mean ± SD of assays (n = 4). **, $P < 0.01$; significant difference from the vehicle-treated cells based on one-way ANOVA followed by a Dunnett's *post hoc* test.

Figure 3 Effects of hPXR-siRNA expression on the activation of CYP3A4 reporter gene and CYP3A4 mRNA levels

A. 3-1-10 cells (5.0 x 10^4 cells/well) in a 6-well plate were infected with AdCont or

AdhPXR-RNAi (MOI of 100). Three days after infection, the cells were incubated with 0.1% DMSO (vehicle), 10 μM RIF, 5 μM CTZ or 80 nM VD_3 for 48 hours. Luciferase activities were determined and are expressed as relative values to vehicle-treated cells infected with AdCont. Data represent the mean ± SD (n = 4). *, $P < 0.05$; **, $P < 0.01$; significant difference from the vehicle-treated cells based on one-way ANOVA followed a Tukey's *post hoc* test. †††, $P < 0.001$; significant difference from the corresponding AdCont-infected cells based on an unpaired Student's *t* test. B. Western blotting was carried out with nuclear extracts (30 μg/lane) prepared from the cells used in A as described in Methods. C. HepG2 cells (1.0×10^5 cells/well) in a 6-well plate were infected with AdCont or AdhPXR-siRNA (MOI of 50) and treated with 0.1% DMSO, 10 μM RIF or 10 nM VD_3 for 48 hours. Total RNAs were prepared from the cells and were subjected to RT-PCR analysis as described in Methods.

Figure 4 Pyributicarb, EPN and isoxathion activate the* CYP3A4 *promoter through PXR

A. 3-1-10 cells (2.0×10^4 cells/well) in a 24-well plate were infected with adenovirus cocktail (MOI of 25). The cocktail titer including AdhPXR-siRNA (MOI of 0 to 25) was adjusted by the AdCont titer. Three days after the infection, these cells were treated with 0.1% DMSO (vehicle), 10 μM RIF, 10 nM VD_3, 3 μM pyributicarb or 10 μM other pesticides, and luciferase activities were determined. The activities are expressed as fold to those in the vehicle-treated cells infected with only AdCont. Data represent the mean ± SD (n = 4 for pesticide) and the mean of duplicates (RIF-treated cells). ††, $P < 0.01$; significant difference from the corresponding

AdCont-infected cells based on one-way ANOVA followed by a Dunnett's *post hoc* test. B. HepG2 cells (1.0 x 10^5 cells/well) in a 12-well plate were infected with AdCont or AdhPXR-siRNA (MOI of 30). Three days after the infection, these cells were treated with 3 μM pyributicarb or 10 μM isoxathion, EPN, or RIF for 48 hours. Total RNA preparation and real-time PCR was carried out as described in Methods. CYP3A4 mRNA levels were normalized by using those of GAPDH. Data represent the mean ± SD (n = 3). *, $P < 0.05$; significant difference from the vehicle-treated cells based on one-way ANOVA followed by a Tukey's *post hoc* test. †, $P < 0.05$; ††, $P < 0.01$; difference from the AdCont-infected cells based on an unpaired Student's *t* test.

Figure 5 Activation of the CYP3A4 reporter gene by pyributicarb in mouse liver

Control mice were infected with AdCYP3A4-362-7.7k (3.4 x 10^9 $TCID_{50}$/mouse) and AdCont (0.66 x 10^9 $TCID_{50}$/mouse), while the hPXR-group mice infected with AdCYP3A4-362-7.7k (3.4 x 10^9 $TCID_{50}$/mouse), AdCont (0.22 x 10^9 $TCID_{50}$/mouse) and AdhPXR (0.44 x 10^9 $TCID_{50}$/mouse) via intravenous injections. Two days after infection, mice were treated with vehicle (0.5% methyl cellulose/saline), pyributicarb (100 mg/kg/day) or PCN (50 mg/kg/day) p.o. for 2 consecutive days. Luciferase activities in the livers were measured as described in Methods, and normalized by protein concentration. Columns and bars represent the mean and SD, respectively (n = 3 or 4).

Table 1. Primer and probe sequences for RT-PCR

	Oligonucleotide sequences
Conventional RT-PCR	
PXR sense	5´-AACACAGTGTTCAACGCG-3´
PXR antisense	5´-GAAGAGGGAGATGGCCTGC-3´
GAPDH sense	5´-AACAGCCTCAAGATCATCAGC-3´
GAPDH antisense	5´-GGATGATGTTCTGGAGAGCC-3´
CYP3A4 sense	5´-TGATTCCAAGCTATGCTCTTCACCG-3´
CYP3A4 antisense	5´-CTGGTTGAAGAAGTCCTCCTAAGCT-3´
Real-time PCR	
CYP3A4 sense	5´-GATTGACTCTCAGAATTCAAAAGAAACTGA-3´
CYP3A4 antisense	5´-GGTGAGTGGCCAGTTCATACATAATG-3´
CYP3A4 probe	5´-FAM-AGGAGAGAACACTGCTCGTGGTTTCACAG-TAMRA-3´
GAPDH sense	5´-GAAGGTGAAGGTCGGAGTC-3´
GAPDH antisense	5´-GAAGATGGTGATGGGATTTC-3´
GAPDH probe	5´-FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3´

Fig. 1A

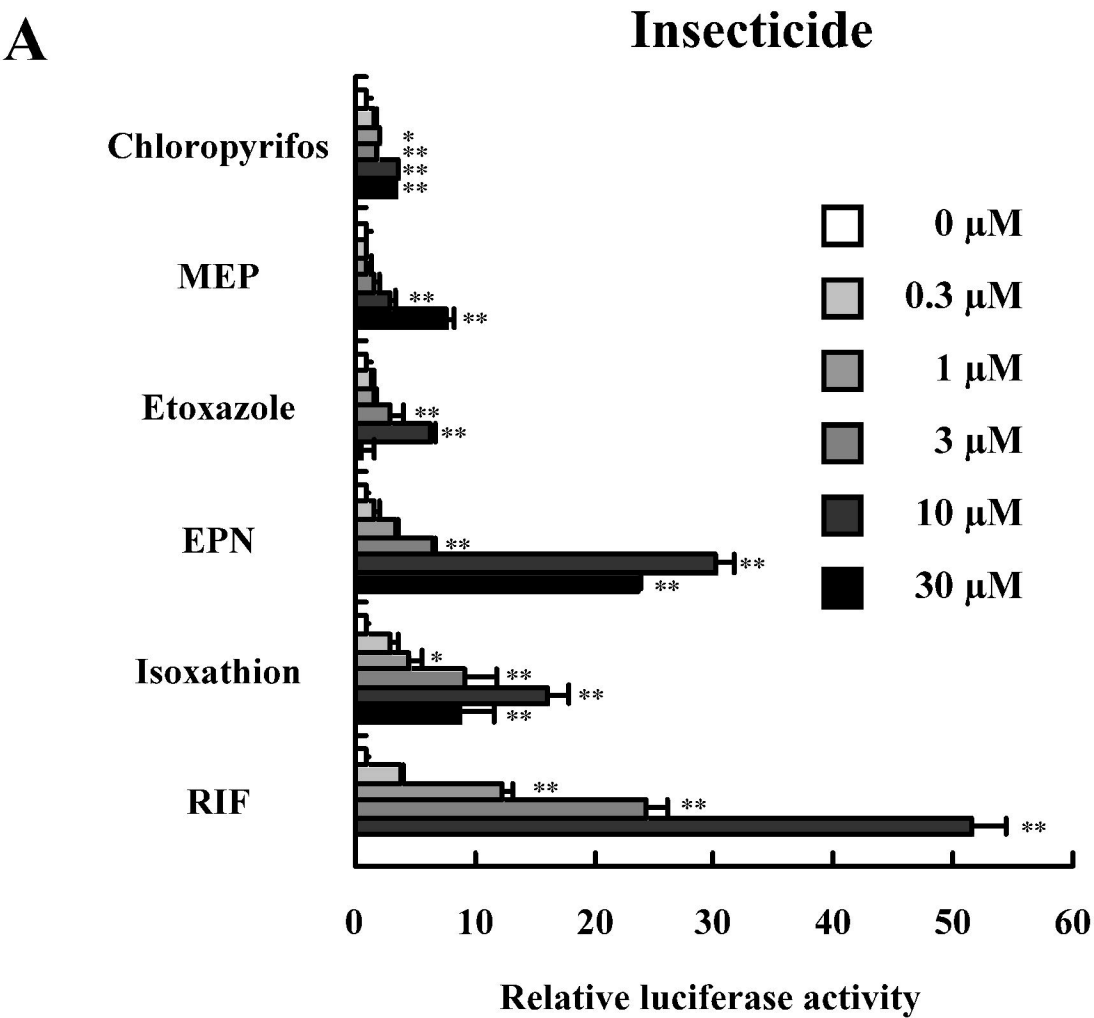
A
Insecticide
Chloropyrifos
MEP
Etoxazole
EPN
Isoxathion
RIF
0 μM
0.3 μM
1 μM
3 μM
10 μM
30 μM
0
10
20
30
40
50
60
Relative luciferase activity

Fig. 1B

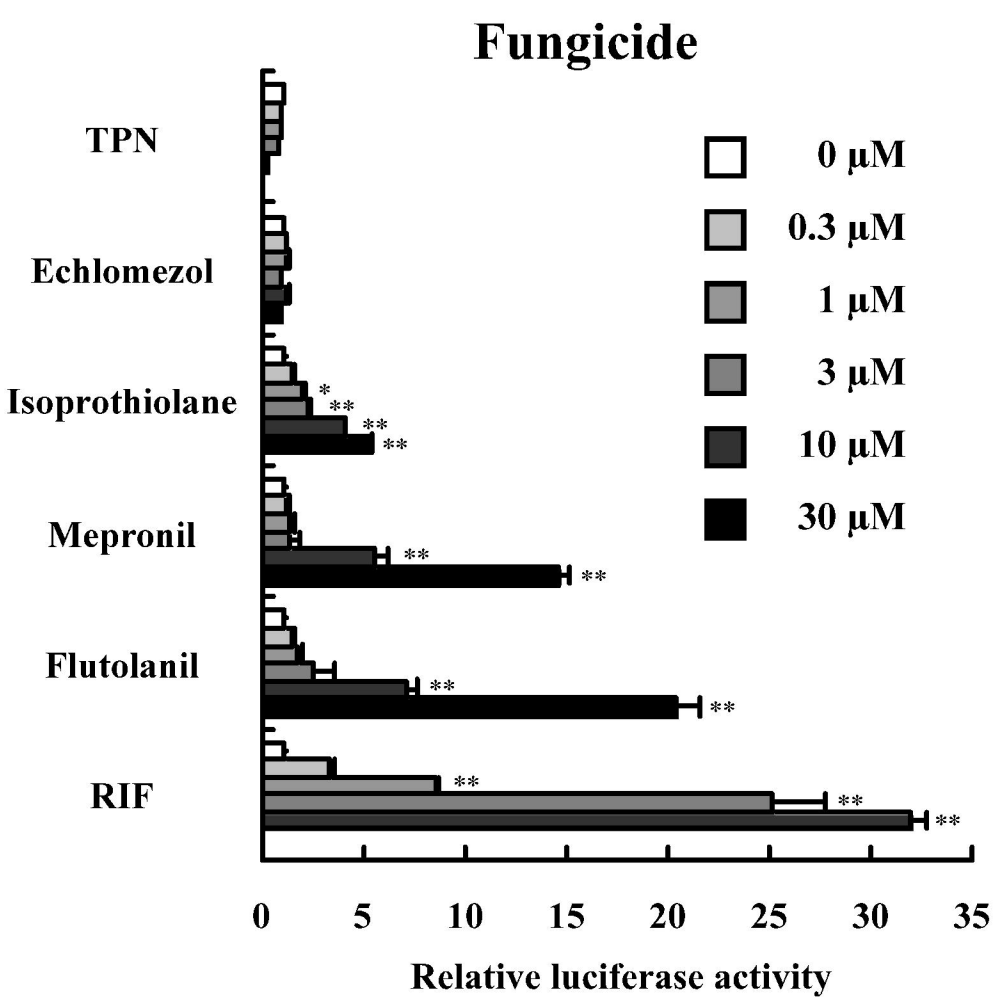
B
Fungicide
TPN
Echlomezol
Isoprothiolane
Mepronil
Flutolanil
RIF
0 μM
0.3 μM
1 μM
3 μM
10 μM
30 μM
*
**
0
5
10
15
20
25
30
35
Relative luciferase activity

Fig. 1C

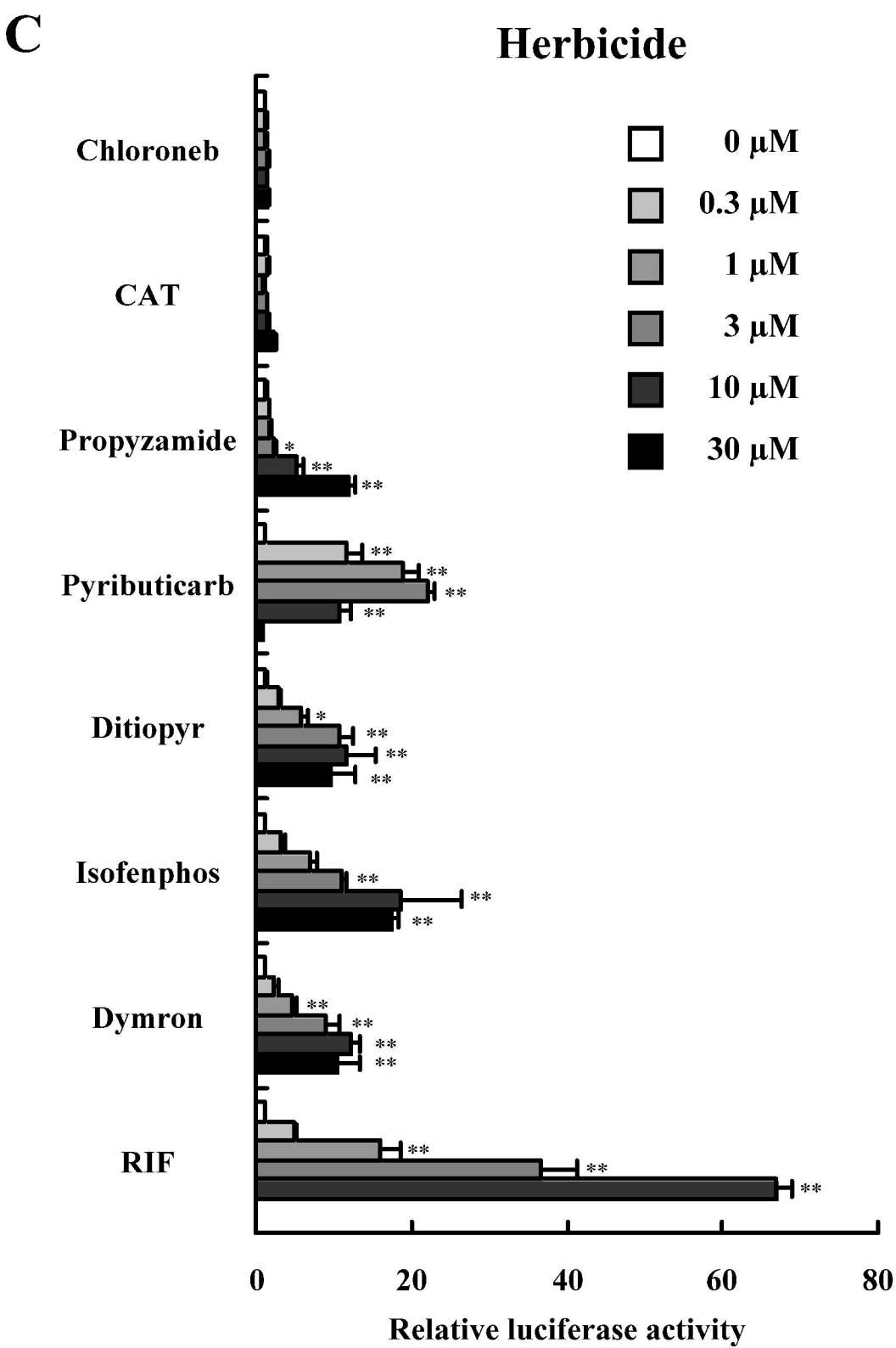
C
Herbicide
0 μM
0.3 μM
1 μM
3 μM
10 μM
30 μM
Chloroneb
CAT
Propyzamide
Pyributicarb
Ditiopyr
Isofenphos
Dymron
RIF
0
20
40
60
80
Relative luciferase activity

Fig. 2

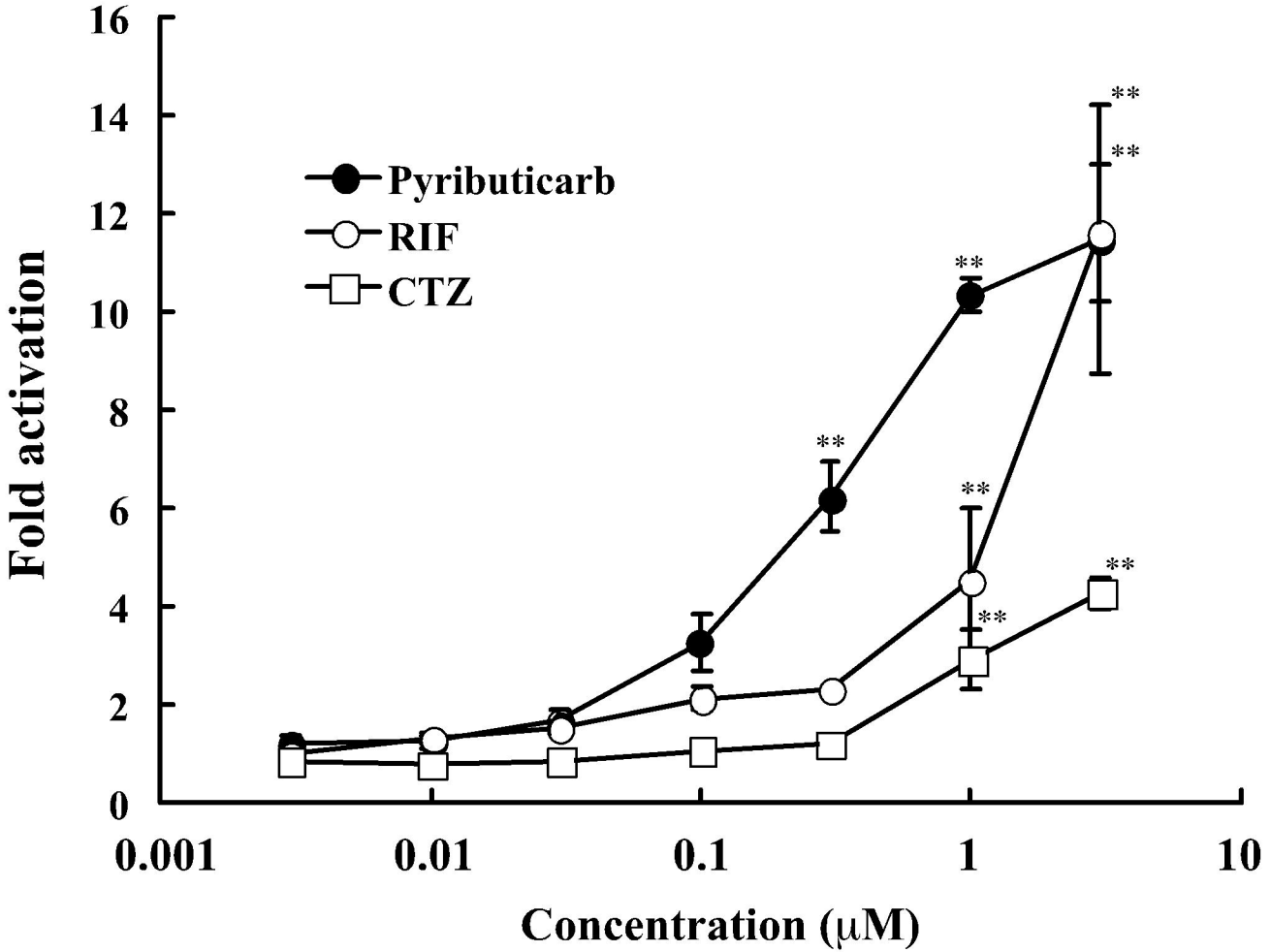
16
14
12
10
8
6
4
2
0
Fold activation
Pyributicarb
RIF
CTZ
**
**
**
**
**
**
0.001
0.01
0.1
1
10
Concentration (μM)

Fig. 3

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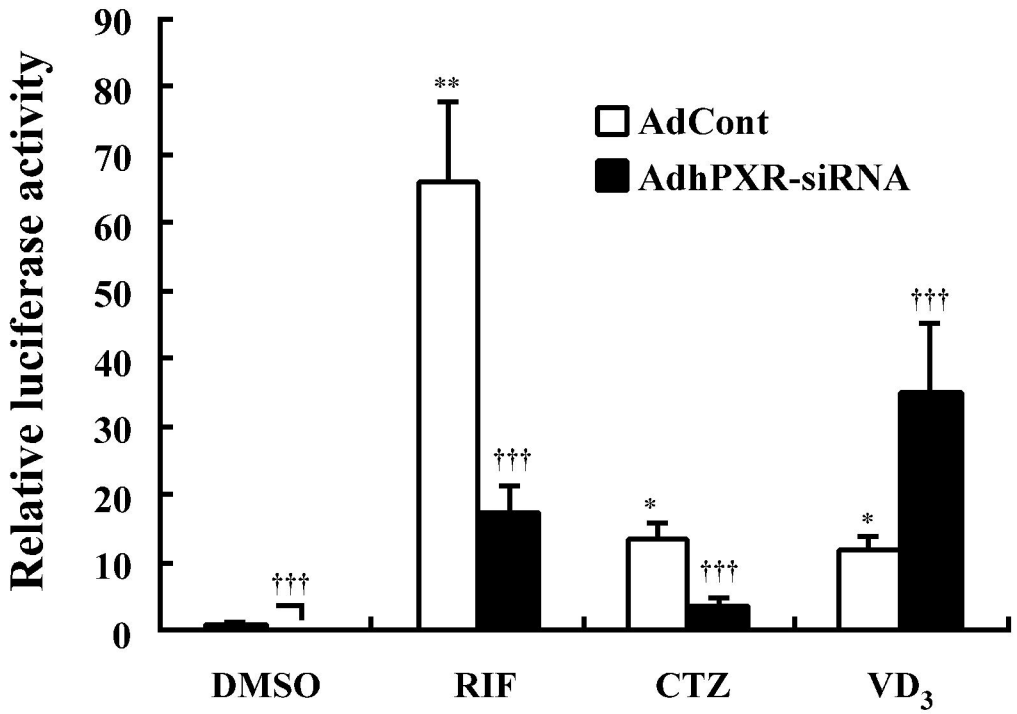
A



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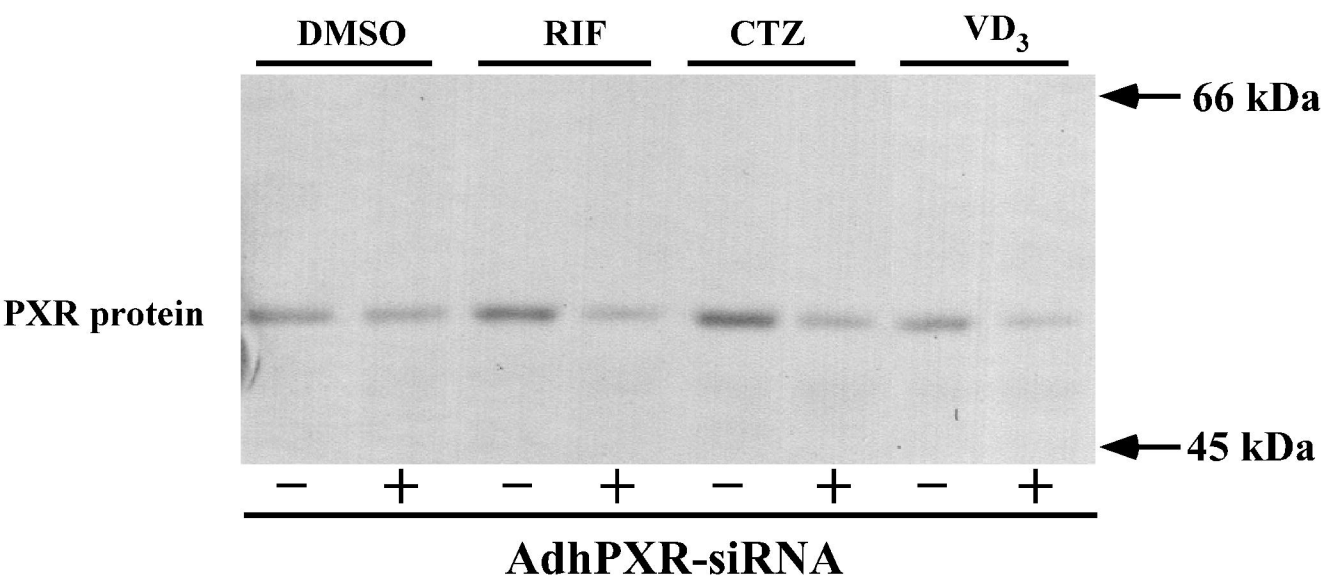

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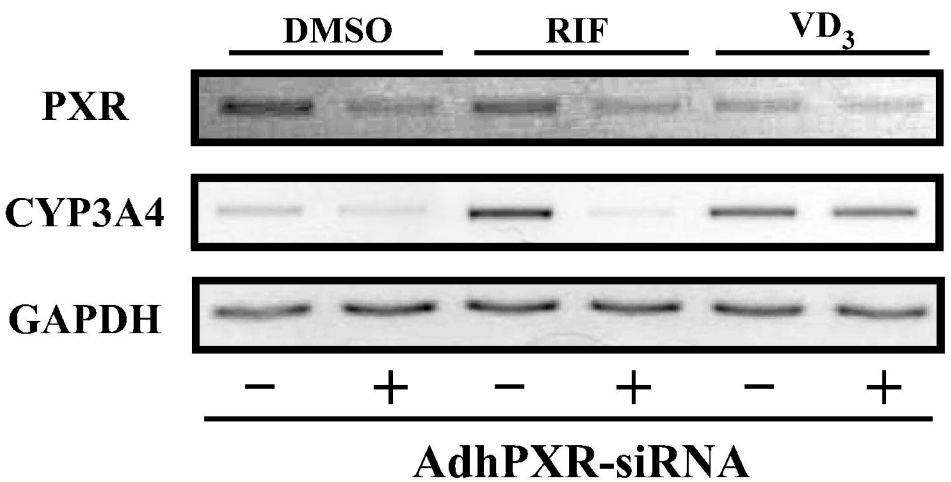

Fig. 4

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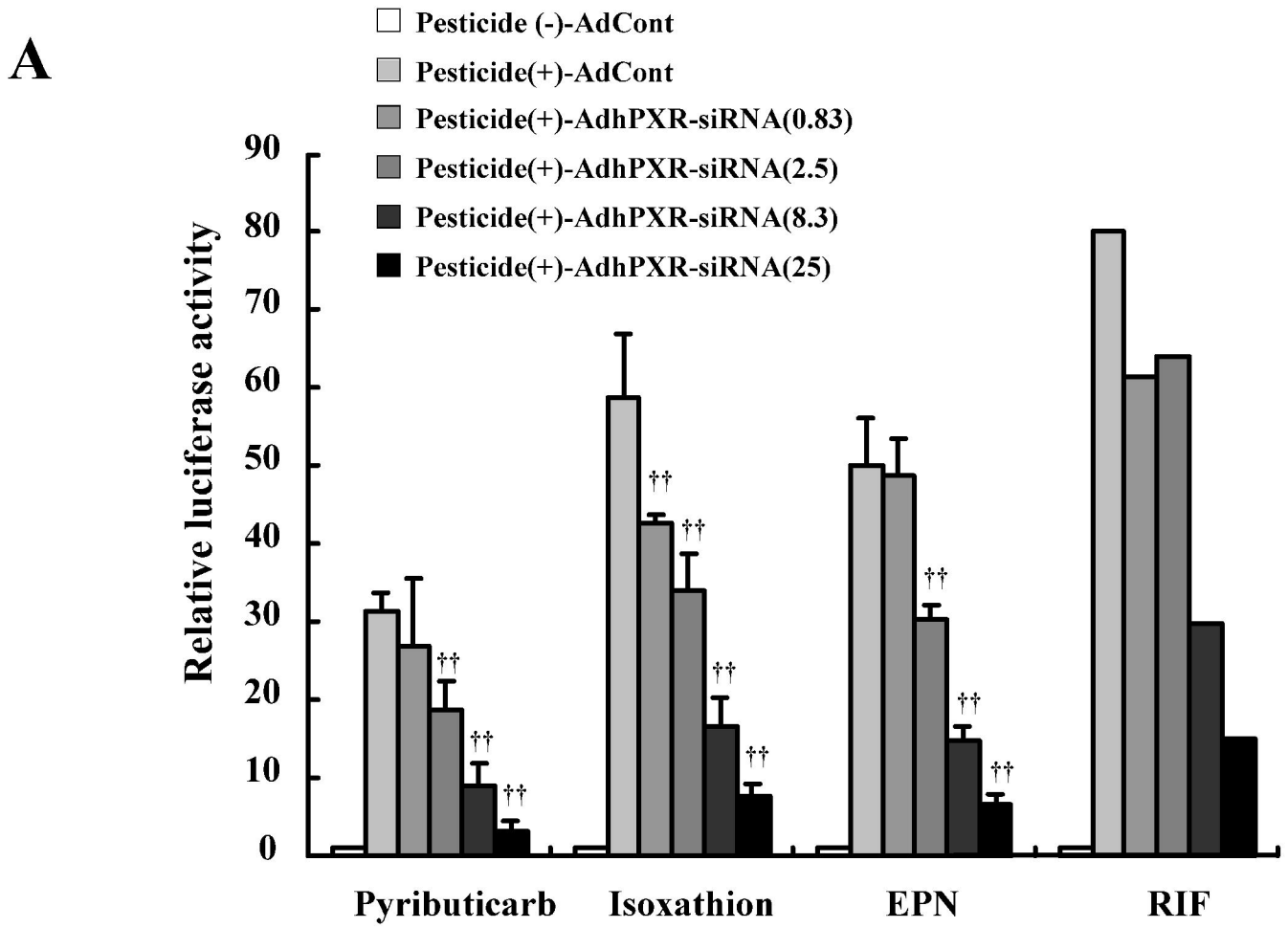


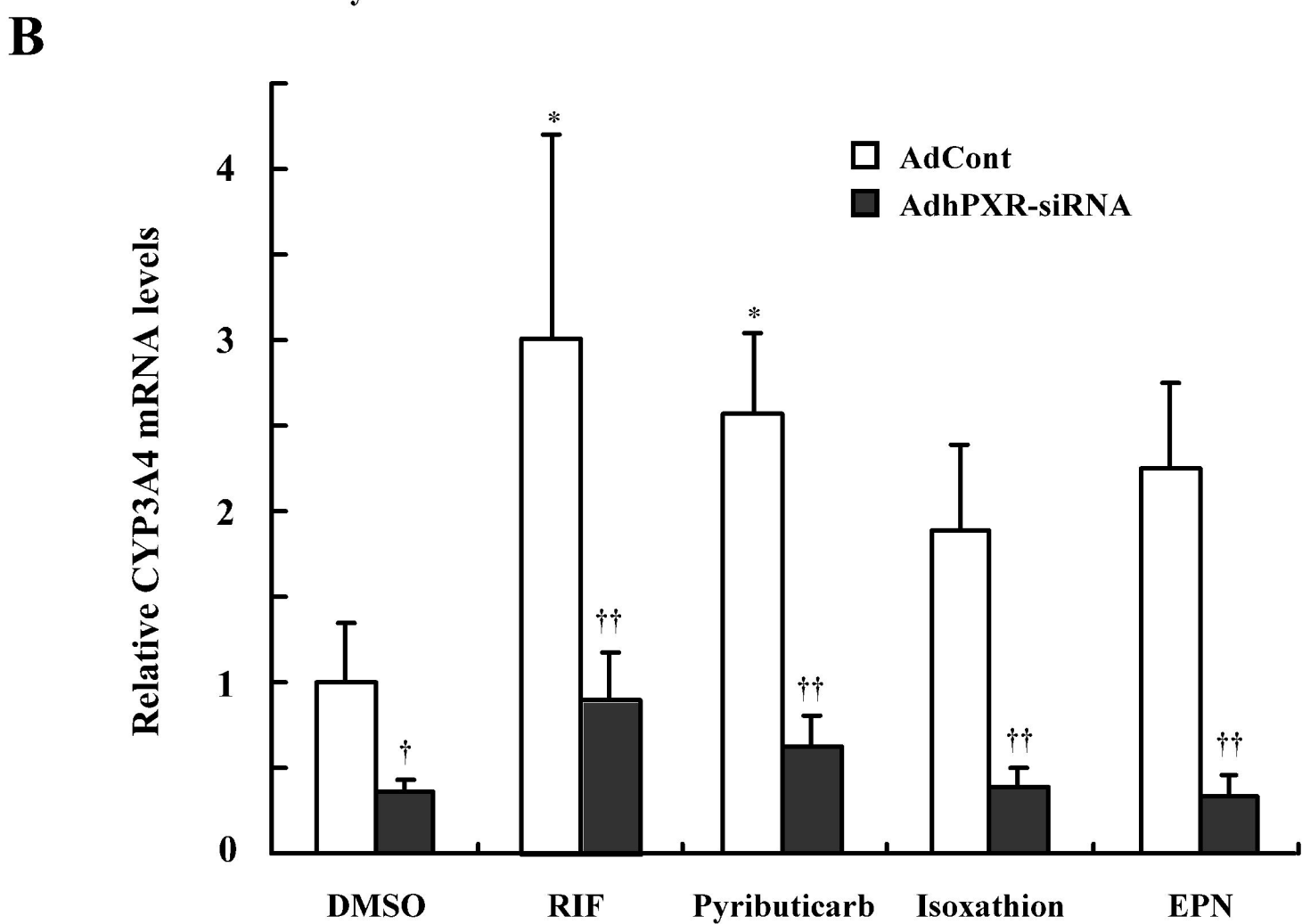
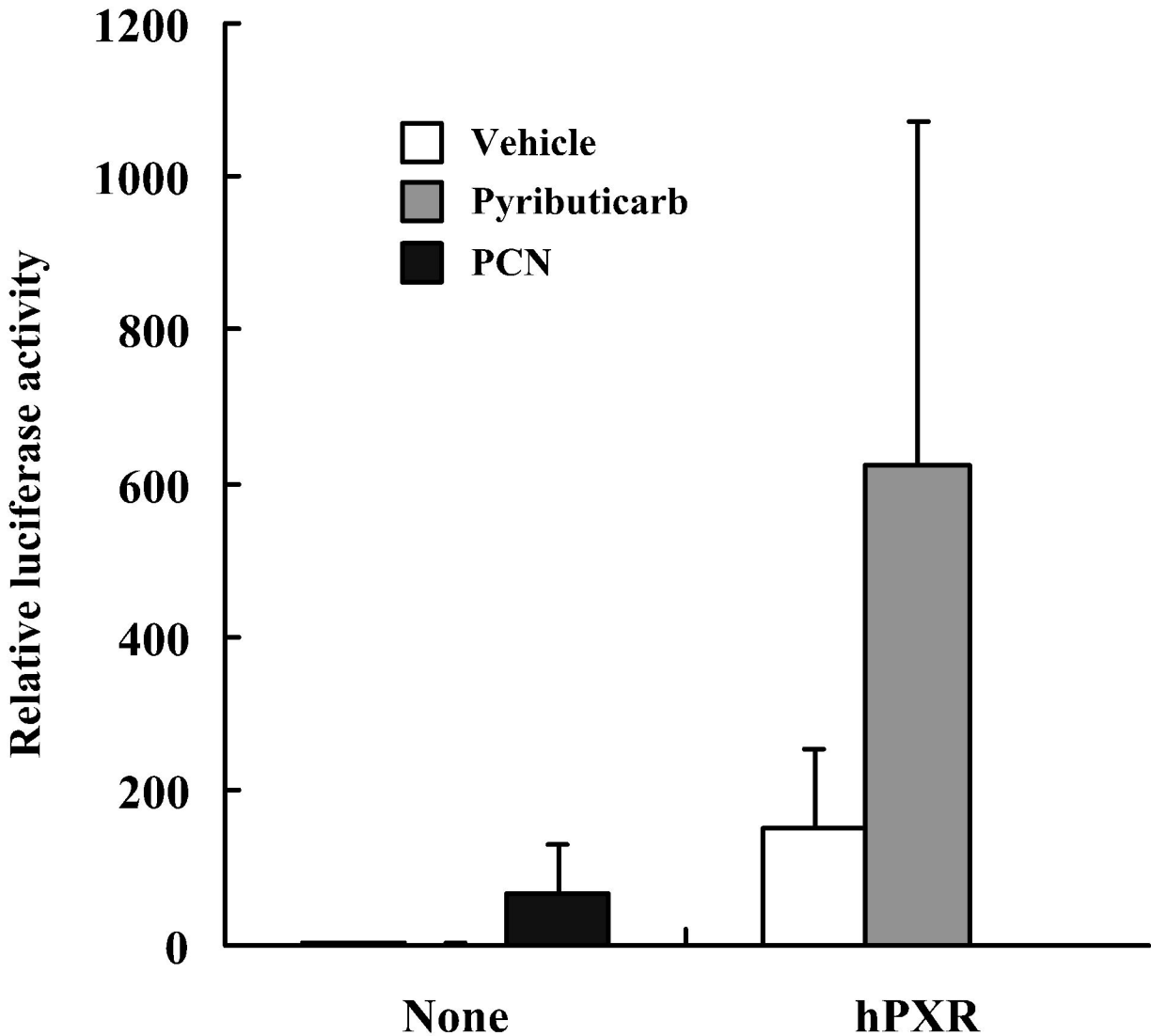


Fig. 5


1200
1000
800
600
400
200
0
Relative luciferase activity
Vehicle
Pyributicarb
PCN
None
hPXR