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PXR-dependent induction of human *CYP3A4* gene expression by organochlorine pesticides

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Abstract

OCP are xenobiotics which display various toxic effects on animal and human health. One of their effects is to bind and activate estrogen receptor alpha (ERα). We have previously studied the down-regulation of induced *CYP1A1* (cytochrome P450) expression by this class of molecules in mammary carcinoma cells and shown the importance of ERα in this process. However, an alternative mechanism was suggested by those experiments in hepatoma cells. In this study, we have performed Northern blot and transient transfection assays in various cell lines and shown that OCP activate human pregnane X receptor (PXR) and subsequent CYP3A4 mRNA expression. This effect is mediated by the distal xenobiotic responsive element modulator of the promoter. The induction of *CYP3A4* by OCP was dose-dependent within the 1–10 μM range. The data suggest that chronic exposure to OCP could alter a major metabolite pathway in human liver and putatively modify the pharmacokinetics of drugs and pollutants.

Keywords: Cytochromes P450; Gene expression; Organochlorine pesticides; PXR; Liver metabolism; Xenobiotic

1. Introduction

OCP are produced by industrial processes and used during agricultural procedures. They contaminate the food chains and display toxic effects in animals and human populations. For example, dieldrin, a commonly used insecticide, is suspected to increase breast cancer risks [1]. Indeed, most OCP display estrogenic actions because they bind and activate ER [2]. Moreover, estrogen exposure is known to increase breast cancer risk [3]. As a consequence, these xenobiotics are called xenoestrogens.

Activation of ERα leads to an increase of the transcription of a plethora of genes and in the repression of other genes [4]. We and other groups have previously shown that E2 inhibits *CYP1A1*-induced transcription [5]. CYP1A1 is an enzyme involved in xenobiotic metabolism and belongs to the CYP superfamily best known as major phase I xenobiotic metabolizing enzymes (XMEs). Expression of the *CYP1* (1A1, 1A2, 1B1) subfamily is induced by dioxins, furans and polychlorinated biphenyls and this effect depends on the activation of the aryl hydrocarbon receptor (AhR) transduction pathway [6]. Inhibition of dioxin-mediated *CYP1A1* activation in mammary carcinoma cells depends on ERα. As a consequence, we have studied the effects of xenoestrogens on this gene and shown that these xenobiotics display the same actions as natural estrogens [5]. However, in the hepatoma cell line, HepG2, which does not contain functional ERα, OCP but not E2 repress *CYP1A1* gene expression [5]. This result shows that other transduction pathways could be involved in xenobiotic effects.

Recent studies have shown that some OCP could activate the PXR, a new member of the nuclear hormone receptor superfamily [7,8], which is involved in *CYP3A* transcription in the liver and the gut [9,10]. These studies have shown an activation of rodent PXRs (rat and mouse) and subsequent *CYP3A23* (mouse) and *CYP3A1* (rat) induction [9,10]. Binding of pesticides to rodent PXR required the ligand binding domain (LBD) which is poorly conserved in the human gene [11–14]. As a consequence, some ligands (rifampicin) of the human PXR do not bind rodent PXR

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Abbreviations: CYP, cytochrome P450; E2, 17β-estradiol; ER, estrogen receptor; FL, firefly luciferase; OCP, organochlorine pesticides; PXR, pregnane X receptor; PXRE, PXR responsive element; tk, thymidine kinase; XREM, xenobiotic responsive element modulator.

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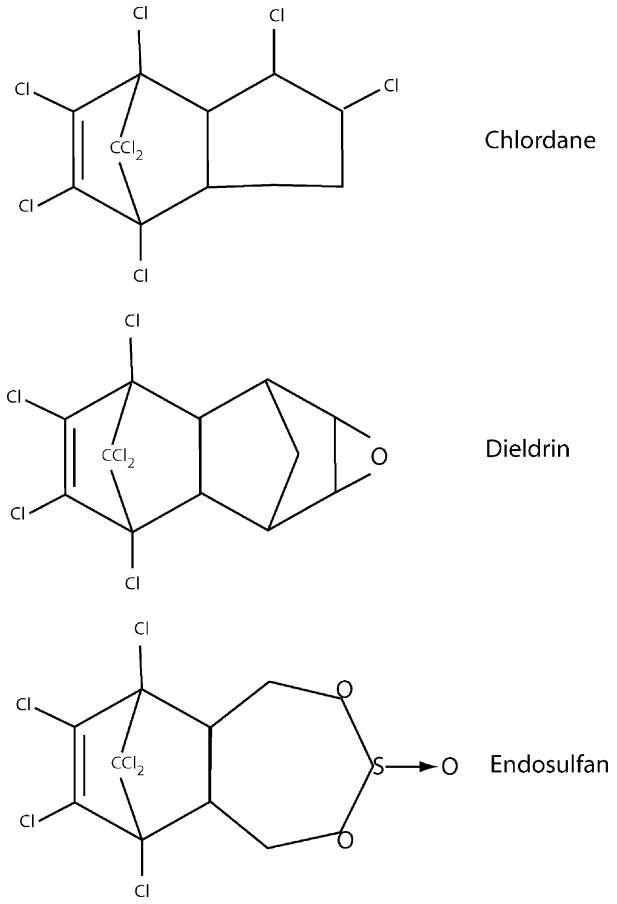

Fig. 1. Structures of the three pesticides used in this study.

[15]. Here, we show that three OCP, α-endosulfan, chlordane and dieldrin (Fig. 1), activate human PXR and *CYP3A4* transcription and could thus alter the metabolism of a large number of endogenous as well as xenobiotic compounds.

2. Material and methods

2.1. Chemicals

Chlordane, dieldrin and α-endosulfan were obtained from Promochem and dissolved in ethanol (10 mM, volume of treatment 0.1%). The certified value of purity of these pesticides is 64.78, >99 and >99%, respectively. Other chemicals were obtained from Sigma (unless otherwise stated) and oligonucleotides were from Genset. DMEM culture medium was purchased from Invitrogen.

2.2. Cell culture

The human hepatoma cell line HepG2 (ATCC) and the human mammary tumor cell line MCF-7 (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies) with phenol red and supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies), and 0.5 μg/mL fungizone (Squibb). Primary cultures of human hepatocytes were prepared and cultured as described by Gerbal-Chaloin *et al.* [16].

2.3. Northern blots

Total RNA preparation was performed with RNA Easy Midi Kit (Qiagen). Northern blots were performed as already described [5]. Probes were synthesized from cDNAs with the Megaprime DNA labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.4. Plasmids

The Firefly luciferase (FL) expression plasmid (pGL3 basic vector) was purchased from Promega. CDG1, SXR (human PXR) and $(PXRE)_3$-tk-LUC expression or reporter vectors were provided by R. Evans and are described by Blumberg *et al.* [11]. CYP3A4 reporter vectors were subcloned following the instructions of Goodwin *et al.* [7]. Ligation experiments were performed with T4 DNA ligase (Invitrogen).

Plasmids were checked by DNA sequencing analysis and amplified using the Qiagen Maxiprep Kit.

2.5. Transfection experiments

Transfection experiments were performed in HepG2 cells as described previously [5]. Briefly, 1 day prior to the transfection, cells (0.3×10^6 cells/6-cm dish) were seeded into DMEM with phenol red, 10% fetal calf serum, penicillin, streptomycin and fungizone (see Section 2.2 for details). The luciferase reporter vectors (1 μg of DNA) and the expression vectors (0.1 μg of DNA) were introduced into the cells by the calcium phosphate co-precipitation technique followed by a 2-min glycerol shock. Twenty four hours after transfection, cells were treated by adding various agents to the culture medium. After an overnight incubation, cells were homogenized for enzymatic assays with phosphate lysis buffer (Promega). Luciferase assays (Firefly) were performed with a Promega kit and a luminometer. Blanks were obtained by assaying luciferase activity in mock-transfected cells.

MCF-7 cells were maintained in DMEM (Life Technologies) with phenol red and supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies) and 0.5 μg/mL fungizone (Squibb) and seeded into DMEM without phenol red (Life Technologies) supplemented with 10% charcoal-treated calf serum (from which steroids had been removed), and the same concentrations of penicillin, streptomycin, and fungizone as described above. They were treated and homogenized like HepG2 cells. Transfections were performed as follows: each dish was transfected with 500 μL of medium containing 3 μL of DAC-30 solution (Eurogentec) mixed with 1 μg of pFL (3A4 constructs or tk-LUC constructs)

and/or 0.1 μg of expression vectors. After 4 hr, the medium was removed and replaced by fresh medium.

3. Results

Human PXR is most expressed in liver and intestine, two major detoxication organs. We used human hepatocytes to evaluate the effect of pesticides (Fig. 1) and 17β-estradiol (E2) on *CYP3A4* expression. Fig. 2 shows that treatment of cultured human hepatocytes with α-endosulfan (10 μM) for 24 hr increases CYP3A4 mRNA levels. In contrast, 17β-estradiol (E2) (10 nM) had no effect. This observation shows that although E2 and α-endosulfan display similar effects on *CYP1A1* regulation, they exert a distinct action on *CYP3A4* regulation in human hepatocytes.

We then performed transient transfection assays in HepG2 cells, a hepatoma-derived cell line. We used an expression vector of human PXR, also known as SXR and two constructs of the *CYP3A4* gene promoter described by Goodwin *et al*. The first construct contains the proximal promoter of human *CYP3A4* gene (UP1) which includes a everted repeat (ER6), a previously described PXR response elements. The second one contains the same proximal promoter with an upstream xenobiotic response element

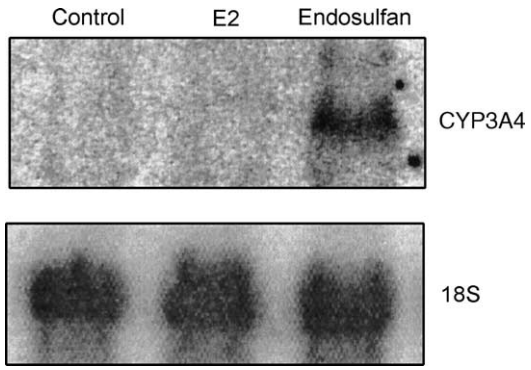

Fig. 2. Effect of α-endosulfan and E2 in human hepatocytes. Total RNA from human hepatocytes were prepared as described in Section 2. Cells were treated with 10 nM of E2 or 10 μM of α-endosulfan for 24 hr before harvesting. Northern blot was performed as already described. Hybridizations were performed with CYP3A4 and 18S probes.

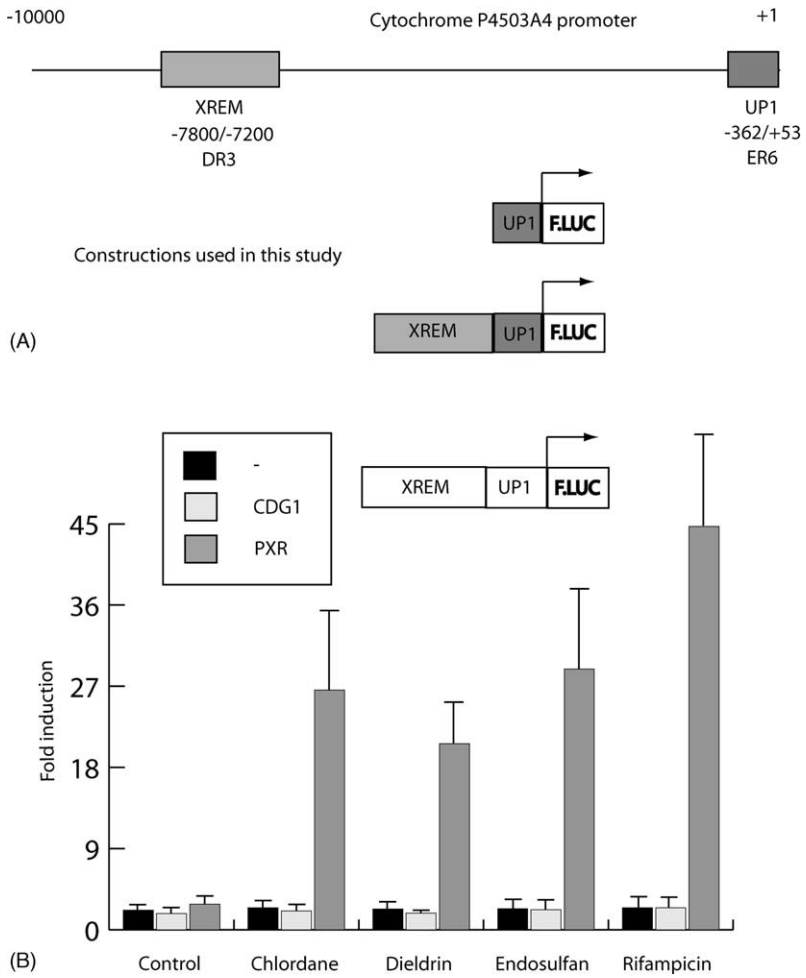

Fig. 3. PXR-dependent induction of the *CYP3A* gene promoter. (A) Structure of the CYP3A4 promoter and of the constructs used in this study. (B) HepG2 cells were transfected with the XREM-UP1 construct in the absence or presence of either CDG1 (control) or CDG1-PXR. Cells were treated with 10 μM of chlordane, dieldrin or α-endosulfan or 50 μM of rifampicin for 24 hr. Firefly luciferase (FL) activities were assayed and fold induction was calculated for each condition. Data shown are the means ± SE (bars) of three independent determinations obtained in three series of experiments.

modulator called XREM which is required to observe a potent induction of the reporter gene (UP1-XREM) (Fig. 3A). Fig. 3B shows that the organochlorine pesticides, chlordane (10 μM), dieldrin (10 μM) and α-endosulfan (10 μM) and a typical PXR ligand, rifampicin (50 μM), induce UP1-XREM-mediated transcription up to 20-fold only in the presence of PXR. In contrast, the proximal construction did not respond to organochlorine pesticides with or without PXR (data not shown). This result confirms that pesticides activate PXR and *CYP3A4* transcription and that the XREM is required for the induction as previously shown [7]. Obviously, the UP1 promoter is not sufficient to mediate even a partial induction in the HepG2 cells.

In order to test the cell and promoter specificity of the effect of these pesticides on PXR responsive genes, we performed dose–response studies using a construct containing three PXRE fused to a tk promoter and a luciferase reporter gene in human MCF-7 cells which do not contain endogenous PXR. Fig. 4 shows that OCP had no effect on luciferase activity when the cells are not co-transfected with a PXR expression vector. However, when PXR is added to the cells, induction with OCP was detected at

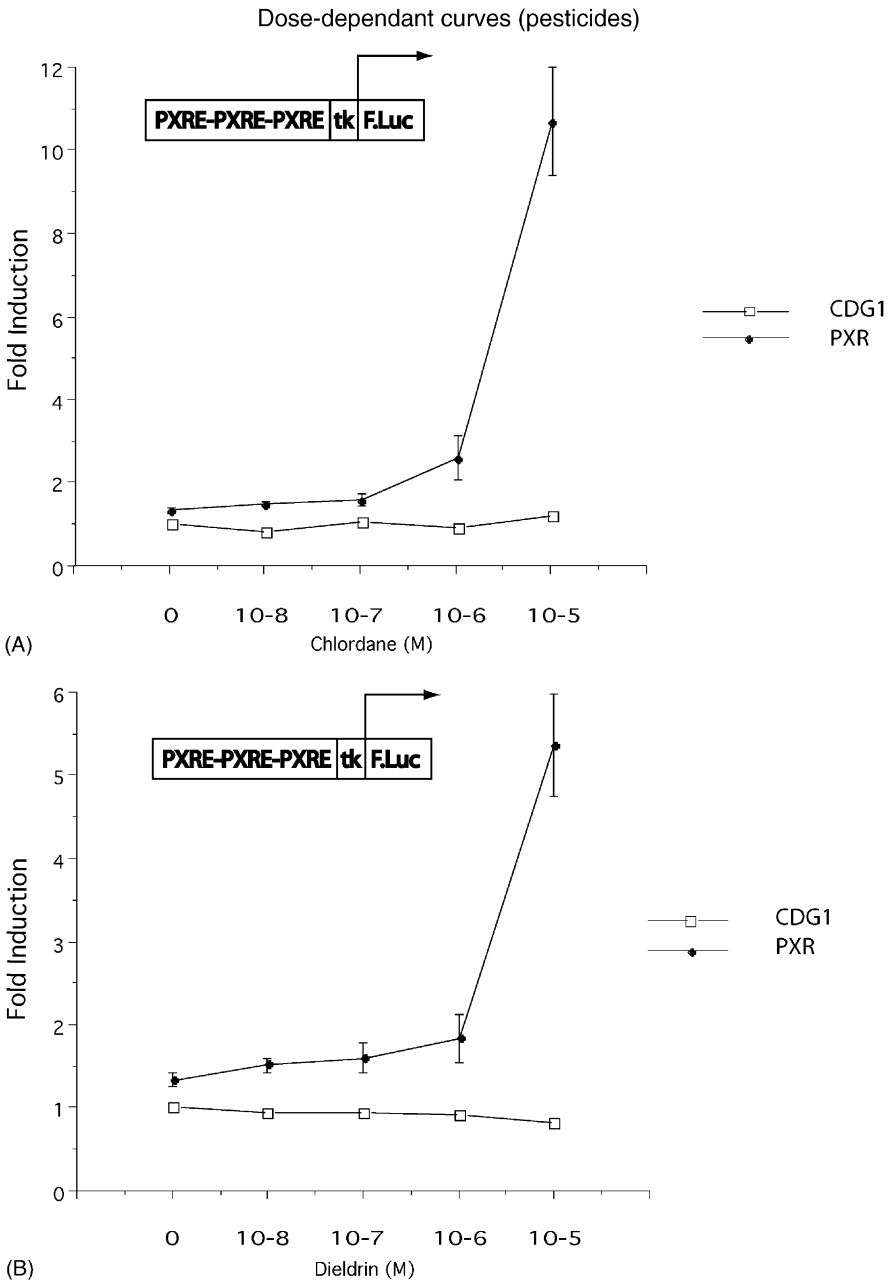

Fig. 4. Dose–response studies in MCF-7 cells. MCF-7 cells were transfected using the DAC-30 method with a $(PXRE)_3$-tk-LUC construct (a kind gift from R. Evans) and co-transfected the CDG1-PXR expression construct (or the control vector CDG1). Inductions were performed with various concentrations of pesticides (A: chlordane, B: dieldrin, C: endosulfan, D: rifampicin). Firefly luciferase activities were tested and fold inductions are represented. Data shown are the means ± SE (bars) of three independent determinations obtained in three series of experiments.

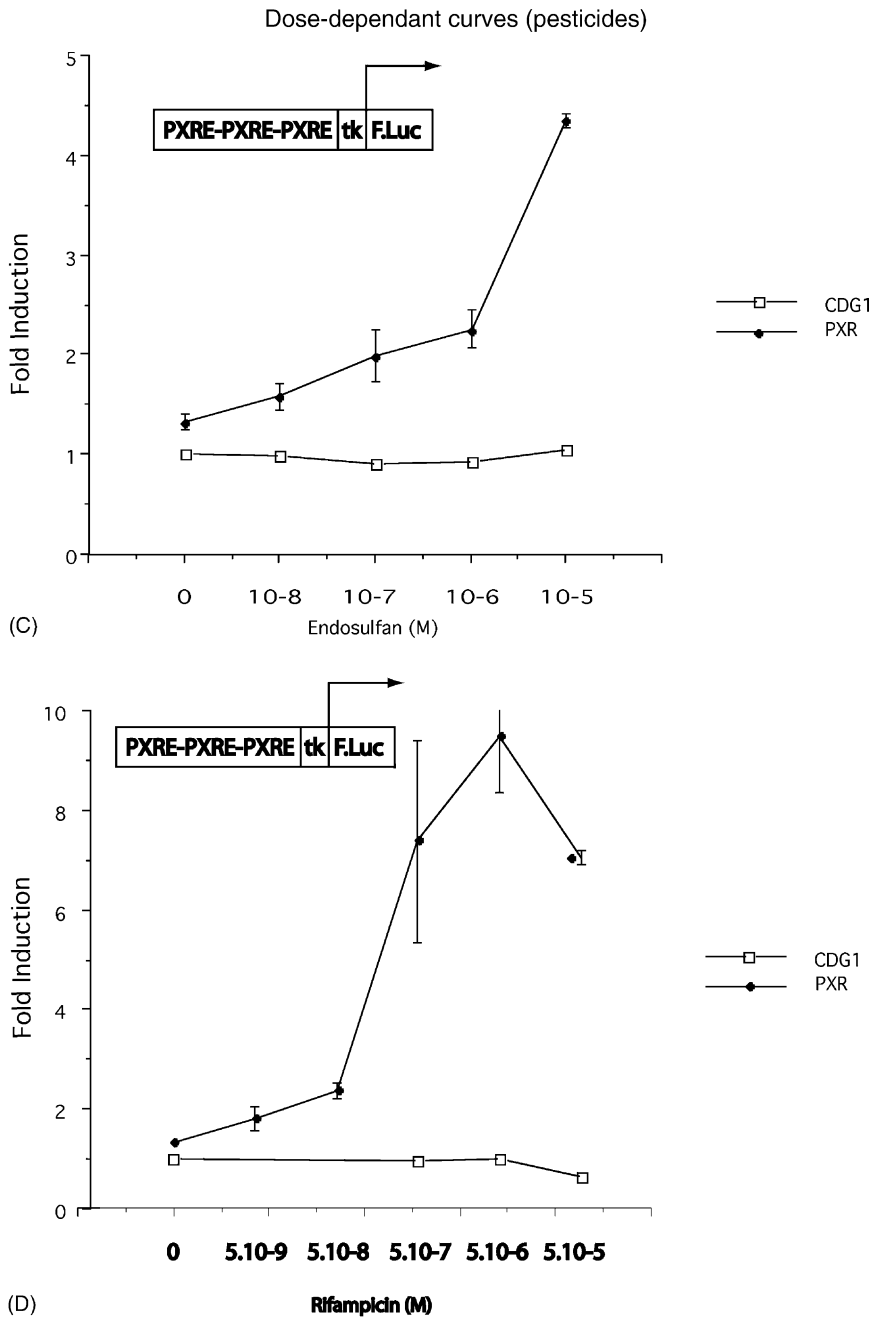

Fig. 4. (*Continued*).

1 μM concentration and was maximal at 10 μM. The pesticides displayed similar dose–response curves. They could not be used at concentrations higher than 10 μM because of their cellular toxicity and therefore it was difficult to determine the concentration giving a half-maximal effect. It is clear that this concentration is higher than that of rifampicin which is one of the most potent inducer of PXR. Nevertheless, the fold activation of this promoter is similar for the four drugs at 10 μM. In fact, the fold activation by chlordane at 10 μM was even higher than that of rifampicin at 5 or 50 μM. Interestingly, the pattern of the dose-dependent activation of PXR by OCP is similar to the one reported for the ER. Indeed, the three OCP used in this study activate the ER and the PXR within the same concentrations range. Thus, those compounds should be considered as both PXR and ER activators.

4. Discussion

Our data provide novel evidence for the activation of human PXR by organochlorine pesticides and subsequent activation of *CYP3A4* gene expression. These observations show that *CYP1A1* and *CYP3A4* are regulated in opposite ways by pesticides. Indeed, *CYP1A1* is down-regulated by these compounds in the MCF-7 and the HepG2 cell lines

whereas *CYP3A4* gene expression is up-regulated in the HepG2 cell line as well as in human hepatocytes. We have demonstrated that the human PXR but not the ER is required to observe the latter effect.

The amounts of pesticides used in this study (1–10 μM range) are higher than those usually detected in the blood of contaminated people. In one study, intoxications of endosulfan above 1 μM have been reported and were correlated with toxic effects [17]. In other studies, lower concentrations of pesticides have been detected in blood and were nevertheless correlated with deleterious consequences such as increased incidence of breast cancer [1]. However, it should be mentioned that those compounds tend to accumulate in fat-rich tissues such as breast. Furthermore, human exposure is usually chronic which could exacerbate the toxic effects of these xenobiotics.

Cytochrome P4503A4 displays a variety of metabolic functions in the liver and the gut [18]. One of these functions is to catabolize E2 leading to the catechol, 2-OH-E2 [19]. This metabolite is not considered as highly toxic but its estrogenic activity is poor [20]. Thus, exposure of liver cells to pesticides would lead to a decrease in the activity of endogenous estrogenic compounds. This may seem paradoxical since the organochlorine pesticides display xenoestrogenic activity. In fact, this intrinsic xenoestrogenic activity which is related to the ability of OCP to bind to the ER is putatively accompanied by a decrease in endogenous estrogens. Another interesting consequence is the cell specificity of pesticides effects. Indeed, in the mammary gland, *CYP1A1* and *CYP1B1* are predominantly expressed while *CYP3A4* is poorly expressed [21]. The effect of pesticides in those cells is primarily to inhibit *CYP1A1* induction [5] which may lead to a decrease in E2 metabolism. In contrast, *CYP3A4* is the predominant *CYP* in the liver. Thus, in this tissue, the most likely effect of pesticides is to increase E2 catabolism.

Induction of *CYP3A4* could have other deleterious effects on human health. Indeed, this enzyme is implicated in more than 60% of drug metabolism [18]. Cross-reactions with drugs, such as oral contraceptives, could be provoked by chronic exposure to organochlorine pesticides as previously hypothesized with hyperforin [22]. Moreover, *CYP3A4*, in association with *CYP1A1* and *CYP2E1*, is a major ROS producer because of uncoupling mechanisms [23]. Oxidative stress is implicated in several human pathologies [24] and pesticides may favor ROS production by activating *CYP3A4* expression. Finally, constitutive expression of PXR in the mouse has been shown to lead to deleterious developmental defects [25]. One hypothesis is that a subsequent induction of *CYP3A23* could alter liver metabolism of critical developmental factors like steroid or other endogenous molecules. It is possible that chronic exposure to pesticides could lead to a similar effect.

In summary, we showed that several organochlorine pesticides activate human PXR and induce *CYP3A4* expression in the liver. The effects of these xenobiotics are usually studied in estrogen-dependent tissues because of ER activation. Our results support the presence of a new transduction pathway, the PXR pathway. Both the ER and the PXR pathways are activated by OCP within a similar range of concentrations. The observations raise the question of OCP toxicity in the liver and in the intestine, two major sites of expression of the promiscuous human PXR.

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References

[1] Hoyer AP, Grandjean P, Jorgensen T, Brock JW, Hartvig HB. Organochlorine exposure and risk of breast cancer. Lancet 1998;352:1816–20.
[2] Massaad C, Barouki R. An assay for the detection of xenoestrogens based on a promoter containing overlapping EREs. Environ Health Perspect 1999;107:563–6.
[3] Wolff MS, Collman GW, Barrett JC, Huff J. Breast cancer and environmental risk factors: epidemiological and experimental findings. Ann Rev Pharmacol Toxicol 1996;36:573–96.
[4] Sanchez R, Nguyen D, Rocha W, White JH, Mader S. Diversity in the mechanisms of gene regulation by estrogen receptors. Bioessays 2002;24:244–54.
[5] Coumoul X, Diry M, Robillot C, Barouki R. Differential regulation of cytochrome P4501A1 and 1B1 by a combination of dioxin and pesticides in the breast tumor cell line MCF-7. Cancer Res 2001;61:3942–8.
[6] Ma Q. Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles. Curr Drug Metab 2001;2:149–64.
[7] Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol 1999;56:1329–39.
[8] Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate *CYP3A4* gene expression and cause drug interactions. J Clin Invest 1998;102:1016–23.
[9] Masuyama H, Hiramatsu Y, Kunitomi M, Kudo T, MacDonald PN. Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate pregnane X receptor-mediated transcription. Mol Endocrinol 2000;14:421–8.
[10] Schuetz EG, Brimer C, Schuetz JD. Environmental xenobiotics and the anti-hormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. Mol Pharmacol 1998;54:1113–7.
[11] Blumberg B, Sabbagh Jr W, Juguilon H, Bolado Jr J, van Meter CM, Ong ES, Evans RM. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev 1998;12:3195–205.

[12] Ekins S, Schuetz E. The PXR crystal structure: the end of the beginning. Trends Pharmacol Sci 2002;23:49–50.
[13] Gillam EM. The PXR ligand-binding domain: how to be picky and promiscuous at the same time. Trends Pharmacol Sci 2001; 22:448.
[14] Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 2001;292:2329–33.
[15] Ekins S, Erickson JA. A pharmacophore for human pregnane X receptor ligands. Drug Metab Dispos 2002;30:96–9.
[16] Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, Maurel P. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. J Biol Chem 2002;277:209–17.
[17] Sancewicz-Pach K, Groszek B, Pach D, Klys M. Acute pesticides poisonings in pregnant women. Przegl Lek 1997;54:741–4.
[18] Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. Ann Rev Pharmacol Toxicol 1999;39:1–17.
[19] Lee AJ, Kosh JW, Conney AH, Zhu BT. Characterization of the NADPH-dependent metabolism of 17beta-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome P4503A4 and 3A5. J Pharmacol Exp Ther 2001; 298:420–32.
[20] Han X, Liehr JG. Microsome-mediated 8-hydroxylation of guanine bases of DNA by steroid estrogens: correlation of DNA damage by free radicals with metabolic activation to quinones. Carcinogenesis 1995;16:2571–4.
[21] Williams JA, Phillips DH. Mammary expression of xenobiotic metabolizing enzymes and their potential role in breast cancer. Cancer Res 2000;60:4667–77.
[22] Wentworth JM, Agostini M, Love J, Schwabe JW, Chatterjee VK. St. John's wort, a herbal anti-depressant, activates the steroid X receptor. J Endocrinol 2000;166:R11–6.
[23] Perret A, Pompon D. Electron shuttle between membrane-bound cytochrome P4503A4 and b5 rules uncoupling mechanisms. Biochemistry 1998;37:11412–24.
[24] Nieto N, Friedman SL, Greenwel P, Cederbaum AI. CYP2E1-mediated oxidative stress induces collagen type I expression in rat hepatic stellate cells. Hepatology 1999;30:987–96.
[25] Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, Evans RM. Humanized xenobiotic response in mice expressing nuclear receptor SXR. Nature 2000;406:435–9.