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Pregnane X Receptor-dependent induction of the CYP3A4 gene by *o,p'*-DDT.

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Running Title: PXR mediates *o,p'*-DDT effects on CYP3A4 transcription

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ABBREVIATIONS: CYP, cytochrome P450; DDT, 1,1,1,-trichloro-2,2-bis (p-chlorophenyl) ethane; PXR, pregnane X receptor; ER, estrogen receptor; GR, glucocorticoid receptor; CAR, constitutive androstane receptor; OCPs, organoclorine pesticides; DMSO, dimethyl sulfoxide; PCN, pregnenolone 16α-carbonitrile; FBS, fetal bovine serum; rtPCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; RT-PCR, reverse transcription- polymerase chain reaction; IL-6, interleukin 6; PBS, phosphate-buffered saline; TLC, thin layer chromatography; BAC, bacterial artificial chromosome; DMEM, Dulbecco's Modified Eagle Medium; HEPES, 4-(2-hydroxyrthyl)-1-piperazineethanesulfonic acid; EDTA,

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ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; PMFS, phenylmethanesulfonyl fluoride; SDS, sodium dodecylsulfate; EMSA, electrophoretic mobility shift assays; TBE, tris base-boric acid-EDTA; ERE, estrogen response element; PXRE, pregnane X receptor response element, GRE, glucocorticoid receptor response element; DDE, 1,1,-dichloro-2,2-bis (p-chlorophenyl) ethylene; DDD, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane; RXRα, 9-cis retinoic acid receptor alpha.

Abstract

Cytochrome P-450 3A4 (CYP3A4), the predominant cytochrome P450 (CYP) expressed in human liver and intestine, contributes to the metabolism of approximately half the drugs in clinical use today. CYP3A4 catalyzes the 6β-hydroxylation of a number of steroid hormones, and is involved in the bioactivation of environmental pro-carcinogens. The expression of CYP3A4 is affected by several stimuli, including environmental factors such as insecticides and pesticides. The *o,p'*-DDT isomer of DDT [1,1,1,-trichloro-2,2-bis (p-chlorophenyl) ethane] comprises approximately 20% of technical grade DDT, which is an organochloride pesticide. We have recently demonstrated that *o,p'*-DDT exposure increases CYP3A4 mRNA levels in HepG2 cells. To determine the mechanism by which *o,p'*-DDT induces CYP3A4 expression, transactivation and electrophoretic mobility shift assays were carried out reveling that *o,p'*-DDT activates the *CYP3A4* gene promoter through the pregnane X receptor (PXR). *CYP3A4* gene promoter activation resulted in both an increase in CYP3A4 mRNA levels and an increase in the total CYP3A4 activity in HepG2 cells. We also observed induction of CYP3A4 and mouse Cyp3a11 mRNA in the intestine of CYP3A4-transgenic mice following exposure to 1 mg/kg of *o,p'*-DDT. At higher doses, a decrease of CYP3A4 inducibility was observed together with an increase in levels of interleukin 6 mRNA, a proinflammatory cytokine that strongly represses CYP3A4 transcription. The present study indicates that regulation of other genes under PXR control may be altered by *o,p'*-DDT exposure.

Introduction

The cytochrome P450 (CYP) gene superfamily encodes a group of heme-containing monooxygenases, many of which metabolize compounds employed as therapeutic drugs. CYPs also play an important role in the oxidation of toxic chemicals and carcinogens, as well as endobiotics such as steroids, prostaglandins and fatty acids (Nelson et al., 1996). The human CYP3A subfamily contains four members, 3A4, 3A5, the predominantly fetal form 3A7, and the relatively poorly understood 3A43 (Nelson et al., 1996; Westlind et al., 2001). CYP3A4 is the most abundant CYP expressed in human liver and small intestine, where it comprises between 50% and 60% of the total CYP, respectively (Kolars et al., 1994; Shimada et al., 1994).

Importantly, CYP3A4 plays a significant role in the metabolism of approximately half of the drugs in use today (Shimada et al., 1994). In addition, CYP3A4 catalyzes 6β-hydroxylation of a number of steroid hormones including testosterone, cortisol, and progesterone (Yamazaki and Shimada, 1997). It is also involved in the bioactivation of environmental procarcinogens, such as aflatoxin B1 (Forrester et al., 1990) and benzo[a]pyrene (Li et al., 1995).

Interindividual variations of up to 40-fold and 17-fold in the expression levels of CYP3A have been observed among human liver and small intestinal specimens, respectively (Shimada et al., 1994, Paine et al., 2006), and a 10-fold variation in metabolism of CYP3A4 substrates *in vivo* has been reported (Thummel and Wilkinson, 1998). This variability is the product of both genetic and environmental factors. CYP3A4 is highly inducible by synthetic glucocorticoids, macrolide antibiotics, phenobarbital, and by environmental contaminants through activation of the estrogen receptor (ER),

glucocorticoid receptor (GR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (Raunio et al., 2005). Therefore, abnormal *CYP3A4* gene expression can result in severe toxicity or therapeutic failure as well as disruption of hormone homeostasis.

Pesticides warrant particular attention since the general human population may be exposed to such chemicals through many routes. 1,1,1,-Trichloro-2,2-bis (p-chlorophenyl) ethane (DDT) is one of the oldest pesticides and its current use is restricted in many countries. However, because of its high efficacy and low mammalian toxicity, it is still used for malaria prevention. Owing to its lipophilic nature and its slow chemical and biological degradation, this molecule tends to be taken up by biological membranes and tissues. It can then become concentrated in organisms and progress up the food chain; thus it is considered a major environmental contaminant. Exposure to DDT alters the activity of hepatic mixed function oxidases and induces expression of CYP2B and CYP3A subfamilies in rodents (Sierra-Santoyo et al., 2000; Wyde et al., 2003). We recently found that *o,p'*-DDT induces CYP3A4 expression in HepG2 cells (Medina-Diaz and Elizondo, 2005).

The ability of the *o,p'*-DDT isomer to act as a ligand to the ER (Forster et al., 1975; Nelson, 1974), suggests that its influence on transcriptional activity may be mediated by this nuclear receptor. On the other hand, recent studies have shown that some organochlorine pesticides (OCPs), including *o,p'*-DDT, can activate the PXR (Coumoul et al., 2002; Lemaire et al., 2004, Lemaire et al., 2006). Therefore, the effects of *o,p'*-DDT on *CYP3A4* gene induction may be exerted through either PXR or ER, or both. Since CYP3A4 induction could be a key determinant of variations in susceptibility to chemical toxicants, it is important to establish whether *o,p'*-DDT alters CYP3A4 expression. The aim of the present study was to evaluate the effects of *o,p'*-DDT, *in vivo*, on CYP3A4

expression as well as to deepen on the knowledge of the molecular mechanism by which this pesticide may alter *CYP3A4* gene expression.

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Materials and Methods

Materials. HepG2 cells were obtained from ATCC (Manassas, VA, USA). The *o,p'*-DDT, trypan blue, rifampicin, 17β-estradiol, ethanol and DMSO were obtained from Sigma-Aldrich (St. Louis, MO, USA). [4-^{14}C]-Testosterone was obtained from Dupont NEN (Boston, MA, USA).

Animals and treatments. Transgenic mice with intestinal expression of CYP3A4 (Granvil et al., 2003) were housed in a pathogen-free facility and fed with autoclaved Purina rodent chow with water available *ad libitum*. All animal manipulations were conducted in accordance with the Mexican Official Norm NOM-062-ZOO-1999 and the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of Health. Animals were randomly distributed into five treatment groups (n = 5). Each mouse received daily gavage doses of corn oil vehicle (control), 50 mg of pregnenolone 16α-carbonitrile (PCN) per kg body weight for 5 consecutive days, or 1, 10 and 100 mg of *o,p'*-DDT per kg body weight for 1 day. Animals were sacrificed by cervical dislocation after the last treatment. The small intestine of the mice were removed, frozen in liquid nitrogen, and stored at -70ºC.

Cell culture. HepG2 cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 μg/ml), 1% L-glutamine, and 1% non essential amino acids (Invitrogen Life Technologies Carlsbad, CA, USA). Cell cultures were maintained in 75 cm^2 flasks at 37ºC, in a humidified incubator with a 5% CO_2 atmosphere.

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Isolation of total RNA. Total RNA was prepared from cultured HepG2 cells or mouse duodenum using the Trizol reagent according to manufacturer's instructions (Invitrogen Life Technologies Carlsbad, CA, USA). RNA was quantified spectrophotometrically at 260 nm and the purity was assessed by measuring OD_{260}/OD_{280} ratio. RNA integrity was evaluated by electrophoresis of RNA samples on 1% agarose gels. cDNA was prepared from 4 μg of total RNA using the SuperScript Pre-amplification System for First Strand Synthesis (Invitrogen Life Technologies Carlsbad, CA, USA) and oligo dT according to manufacturer's instructions.

Real-time quantitative PCR detection. All PCR reagents were purchased from Applied Biosystems (Foster City, CA, USA). Quantitative real-time PCR assay (rtPCR) of the transcripts was performed with gene-specific fluorescent labeled probes in a 7000 Sequence Detector (Applied Biosystems). The probes were labeled with FAM and VIC as the 5'-fluorescent reporter. Non-fluorescent quenchers at the 3'-end of the probes were designed using Primer Express software (Applied Biosystems) and are listed in Table 1. The specificity of the CYP3A4 primers and probe were verified by the lack of amplification from genomic DNA. A standard curve was constructed by serial dilutions of the cDNA of interest as the template. Endogenous GAPDH or 18S, validated for stable expression in HepG2 cells and mouse duodenum, were used for normalization of the mRNA data. The PCR reaction mixture contained 4 μl of cDNA, 1X TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.9 μM and 0.25 μM of primers and probe, respectively. PCR reactions were performed with an ABI PRISM 7000 sequence detector system using the following cycling protocol: 1 cycle of 50ºC for 2 min, 1cycle of 95ºC for 10 min, and 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Each sample was analyzed twice in three

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independent experiments. The results were analyzed using the comparative Ct method as described previously (Livak and Schmittgen, 2001).

RT-PCR analysis. Total RNA from mouse duodenum was isolated by homogenizing tissue in a guanidine/phenol solution (Life Technologies, Carlsbad, CA, USA). Reverse transcription was performance using OneStep RT-PCR kit, according to the manufacturer's instructions (Qiagen, Valencia CA, USA). For interleukin 6 (IL-6) and GAPDH the following program was used: denaturation at 94°C for 4 min and 35 cycles of PCR consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Forward and reverse primers were as follows: IL-6, 5´-TGCTGGTGACAACAACGGCC-3´ and 5´-GTACTCCAGAAGACCAGAGG-3´ (product size 298 bp) respectively, and GAPDH, 5´-GAGGGGCCATCCACAGTCTTC-3´ and 5'-CATCACCATCTTCCAGGAGCG-3´ (product size 340 bp) respectively. The PCR products were electrophoresed on 2% agarose gels, and stained with ethidium bromide to visualize PCR amplification products. Relative intensity was determined using Sigma Gel program (Jandel Scientific software, Sigma).

Testosterone Hydroxylation. HepG2 cells were exposed to 10 μM of *o,p'*-DDT for 24 or 48 h. Then, 3.6 nmol of [4-^{14}C]-testosterone dissolved in ethanol was added to cultures. Six hours later, the medium was collected for analysis of testosterone metabolites and cells were rinsed twice with PBS and scraped with a rubber policeman for protein analysis. Testosterone metabolites were assayed as previously described (Waxman et al., 1991) with some modifications. Briefly, 1 ml of ethyl acetate was added to a 0.5-ml aliquot of culture medium and the mixture was vortexed for 1 min. The layers were separated by centrifugation at 1600 g for 5 min and the organic layer was transferred into a clean test

tube. After a second extraction, the organic extracts were combined and the solvent was evaporated under N_2 at room temperature. The products were separated by thin layer chromatography (TLC) using two solvent systems (A: dichloromethane:acetone 4:1 and B: chloroform:ethyl acetate:ethanol 4:1:0.7) and identified by comparison with unlabelled standards. Radioactivity of each metabolite was indirectly determined using densitometric analysis of bands printed on the plate sensitive Typhoon 4000 (Applied Biosystems., Billerica, MA). Protein concentrations were determined by using the Bradford reaction (Bio-Rad Laboratories, Hercules, CA, USA).

***Plasmids*.** The reporter plasmid p3A4-1200 was constructed as follows: a -1200/+98 fragment was generated by PCR amplification using a BAC clone (Sata et al., 2000) containing the *CYP3A4* gene and oligonucleotides (5'-CTCCTCGAGCTGGCTATCTGGGACGCTGTTCTCTTCTCT-3' and 5'-CTCAAGCTTCTCTCTCCTCTGAGTCTTCCTTTCAGC TCT-3'), which introduced *XhoI* and *HindIII* restriction sites. The fragment was then cloned into the *XhoI/HindIII* site of a pGL-3-Basic vector driving a firefly luciferase gene (Promega, Madison, WI, USA). p3A4-1200 construct enclose three ERE and one PXRE (Hashimoto et al., 1993, Goodwin et al., 1999). The integrity of the construction was verified by enzyme digestion and confirmed by sequencing. The p3A4-362(7836/7208 ins) reporter construct (carrying the xenobiotc responsive enhancer module (XREM) region, the proximal promoter (-362/+53) and the luciferase reporter gene) was generously provided by Christopher Liddle (Goodwin et al., 1999). The p3A4-362(7836/7208 ins) vector enclose one PXRE and one ERE motif (Hashimoto et al., 1993, Goodwin et al., 1999). ERα and hPXR expression vectors were

generously provided by Pierre Chambon from the Institut de Génétique et de Biologie Moléculaire et Cellulaire and Ronald Evans from The Salk Institute, respectively.

Transient transfection. HepG2 cells were cultured in Dulbecco's Modified Eagle described above. The transfection of HepG2 cells was performed using a Multiporator eppendorf with hyposmolar buffer (Eppendorf, Westbury, NY, USA). Each sample (3 x 10^6 cells) was resuspended in 800 μl of hypo-osmolar buffer containing 4 μg of the p3A4-362(7836/7208 ins) or the p3A4-1200 vectors. A mixture of 100 ng of pRL-CMV as an internal control and 4 μg of the PXR or ERα expression vector were transferred to 4 mm cuvettes. A single 1200 volts, 100 μsec pulse was delivered to each sample. After electroporation, the cuvettes were incubated at room temperature for 1 min and then the cells were transferred into OPTIMEM medium. The medium was replaced after 3 h with fresh DMEM containing 10% FBS. Twenty four hours after transfection, cells were treated by adding *o,p'*-DDT, rifampicin or 17β- estradiol to the culture medium. After 24 hours of incubation with the applied treatment, the cells were homogenized for enzymatic assays with phosphate lysis buffer (Promega, Madison, WI, USA). Luciferase activity was performed using the Dual-Glo Luciferase assay System (Promega, Madison, WI, USA) and a DT 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). Blanks were obtained by conducting luciferase activity assays in mock-transfected cells. Firefly luciferase activity levels were normalized by comparison with the renilla luciferase activity levels of the pRL-CMV internal control vector from the same culture.

Prepation of Nuclear Extracts. Nuclear protein extracts were isolated according to the protocol of Schreiber et al. (Schreiber et al., 1989). Briefly, HepG2 cells were cultured to approximately 90% confluence and then collected in PBS buffer by scraping. Cells were

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pelleted by centrifugation at 1500 g for 5 min. The pellet was resuspended in 1 ml PBS, transferred into an Eppendorf tube, and pelleted again by spinning for 15 s in a microfuge. PBS was removed and the cell pellet was resupended in 400 µl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMFS) by gentle pipetting. The cells were maintained on ice for 15 min, and 25 µl of 10% Nonidet NP-40 was added and vigorously vortexed for 10 sec. The homogenate was centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in 50 µl of buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMFS) and vigorously rocked at 4°C for 30 min on a shaking platform. The nuclear extract was centrifuged for 5 min at 4°C and the supernatant was frozen in aliquots at 70°C. Protein concentrations were determined by using the Bradford reaction (Bio-Rad Laboratories, Hercules, CA, USA), and the samples were analyzed by SDS-polyacrylamide gel electrophoresis to verify the integrity of the proteins (without evidence of smearing).

Electrophoretic Mobility Shift Assays. For electrophoretic mobility shift assays (EMSA), 10 µg of nuclear extracts were mixed with 10 µl Protein Buffer (1x) (24 mM HEPES, 1 mM EDTA, 8 mM $MgCl_2$, 120 mM KCl, 20% glycerol, 1 mM DTT) containing 2 µg of poly(dI.dC), 1 mM $MgCl_2$ and 1 mM Spermidine. Following a 10-min incubation at 4ºC, 2 µl of ^{32}P-labeled double-strand oligonucleotides (CYP3A4ER6: 5'-ATATGAACTCAAAGGAGGTCAGTGA-3' or CYP3A4ER6-mut: 5'-ATATGTTCTCAAAGGAGAACAGTGA-3', mutations are underlined) were added. After incubating for an additional 10 min at 4ºC, the samples were run on a 6% polyacrylamide gel with 0.5 X TBE buffer. The gel was dried and exposed to X-film autoradiographs.

Statistical analysis. The results are presented as the mean values ± SD. Statistically significance comparison were detected by Student's t test or the Mann-Whitney test as appropriate. The criterion for significance was $p < 0.05$ in all cases.

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Results

o,p'-DDT activates the CYP3A4 gene promoter through PXR and ERα. A reporter construct (CYP3A4-1200) containing 1200 bp of the human *CYP3A4* gene promoter sequence (carrying 3 ER response elements, ERE) linked to a luciferase gene was used to delineate the mechanism by which *o,p´*-DDT influences *CYP3A4* gene expression in HepG2 cells. *o,p´*-DDT at 10 μM increased luciferase activity by about 22%. In comparison, 50 μM rifampicin, a known PXR ligand, increased luciferase activity by about 30% (Fig. 1).

To assess whether the *o,p´*-DDT effects on *CYP3A4* gene expression were mediated by the PXR and/or ERα, HepG2 cells were co-transfected with a PXR or ERα expression vectors and CYP3A4-1200 reporter construct. In the absence of exogenous ligands, increased levels of PXR or ERα did not result in an increase of luciferase activity (Fig. 2, columns 2 and 5). However, addition of *o,p´*-DDT or rifampicine to cell cultures co-transfected with the PXR expression vector resulted in an increase of luciferase activity by about 40% and 80%, respectively (Fig. 2, columns 3 and 4). Cells co-transfected with the ERα expression vector and treated with *o,p´*-DDT or 17β-estradiol resulted in a 70% increase in the luciferase activity (Fig. 2, column 6 and 7). These data establish that *o,p´*-DDT effects, at least in part, are mediated by PXR and ERα.

To determine the role of the XREM that regulates the *CYP3A4* gene through PXR activation, a reporter construct containing the XREM region, the CYP3A4 proximal promoter and the luciferase reporter gene was used (p3A4-362(7836/7208 ins)). HepG2 cells were transfected with the plasmid (p3A4-362(7836/7208 ins)) and treated with *o,p´*-DDT. *o,p´*-DDT increased luciferase activity in a dose-response manner, to a maximum

level that was 3-fold greater than the control value. Rifampicin (50μM) treatment resulted in a 6.5-fold increase in luciferase activity relative to the control value (Fig. 3A). When HepG2 cells were co-transfected with (p3A4-362(7836/7208 ins)) and the PXR expression vector, and treated with 10 μM of *o,p'*-DDT or 50 μM rifampicin a 4- and 9-fold increase in luciferase activity, respectively, was observed when compared to vehicle-treated cultures (Fig. 3B). All over, these results suggest that the *o,p'*-DDT-induced effects on *CYP3A4* gene promoter are mainly mediated by XREM.

EMSAs were performed to examine whether *o,p'*-DDT treatment enhanced the binding affinity of PXR to the ER6 response element. HepG2 cells were treated with 10 μM *o,p'*-DDT or 50 μM rifampicin, and nuclear extracts were subjected to EMSA. ER6-protein complex formation was increased in the nuclear extracts from *o,p'*-DDT treated-cells (Fig. 4A, lane 3) relative to untreated cells (Fig. 4A, lanes 2 and 5) or rifampicin-treated cells (Fig. 4A, lane 4). Competition experiments revealed that formation of the DNA-protein complex was saturated as the presence of an excess of unlabeled ER6 blocked its formation (Fig. 4B, lanes 4-6). When a mutated ER6 probe was used, the DNA-protein complex did not form (Fig. 4B, lane 7). These results suggest that *o,p'*-DDT exposure induces the binding of PXR to the ER6 response element.

CYP3A4 mRNA levels and activity are increased by o,p'-DDT treatment. In order to evaluate whether *CYP3A4* gene promoter activation mediated by *o,p'*-DDT through PXR resulted in increased CYP3A4 mRNA levels, HepG2 cells were transfected with PXR expression vector and treated with 10 μM of *o,p'*-DDT. Treatment with *o,p'*-DDT resulted

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in a 12-fold increase of CYP3A4 mRNA. A maximum 16-fold increase in mRNA expression was observed when treated cells over-expressed the PXR (Fig. 5).

To determine whether *o,p'*-DDT induced CYP3A4 activity, HepG2 cell cultures incubated with 3.6 nmol [4-^{14}C]-Testosterone were treated with 10 μM *o,p'*-DDT for 24 or 48 h and analyzed by TLC (Table 2). Following the 24-h *o,p'*-DDT treatment, increased levels of 6β-hydroxytestosterone and androstenadione metabolites were observed. Following the 48-h *o,p'*-DDT treatment, increased levels of 6β-, 16β-, 2α-hydroxytestosterone and 6-dihydroxytestosterone metabolites were observed.

To examine whether *o,p'*-DDT exposure alters CYP3A4 mRNA expression *in vivo*, young adult male CYP3A4-transgenic mice were exposed to 1, 10, and 100 mg/kg of *o,p'*-DDT and the CYP3A4 and Cyp3a11 mRNA levels in the duodenums of these mice were determined by rtPCR. CYP3A4 mRNA expression was higher in mice exposed to *o,p'*-DDT or PCN than in the control animals (Fig. 6A). Indeed duodenum CYP3A4 mRNA expression was 32-fold higher in the 1 mg/kg *o,p'*-DDT-treated animals than in the controls. Similar results were obtained when mice were treated with 50 mg/kg PCN. However, following 10 mg/kg *o,p'*-DDT treatment, CYP3A4 mRNA levels were increased by 13-fold (although a substantially lower increase than that of the 1 mg/kg dose), and no difference was observed when mice were exposed to 100 mg/kg *o,p'*-DDT. A similar pattern of results was observed for Cyp3a11 mRNA expression (Fig. 6B).

It was previously reported that IL-6 strongly represses CYP3A4 transcription (Muntane-Relat et al., 1995). Others reported that DDT is able to increase the IL-6 levels (Kim et al., 2004). Therefore, IL-6 mRNA levels were examined in duodenum from mice treated with *o,p'*-DDT (Fig. 7). Treatment with *o,p'*-DDT resulted in a moderate increase of

IL-6 mRNA level, with a maximum induction of 1.2-fold relative to the control value. These results may explain, at least in part, the observed drop in CYP3A4 and Cyp3a11 induction.

Discussion

Over the past decade, expression of the *CYP3A4* gene was found to be induced by an array of structurally diverse compounds (Luo et al., 2004). The interaction of some of these compounds with classic steroid hormone receptors suggests that multiple signal transduction pathways may be involved. Given the importance of CYP3A4 in the metabolism of a variety of drugs, an understanding of this phenomenon is crucial in order to minimize the potential for undesirable drug interactions.

We recently found that *o,p'*-DDT treatment increases CYP3A4 mRNA levels in HepG2 cells (Medina-Diaz and Elizondo, 2005). This pesticide is an agonist for ERα and is thus considered an endocrine disrupter chemical with estrogenic activity (Kuiper et al., 1998). Other organochloride pesticides such as dieldrin and chordane have the potential to activate PXR (Coumoul et al., 2002). Both nuclear receptors, ERα and PXR, regulate CYP3A4 expression (Goodwin et al., 1999, Tsuchiya et al., 2005). To determine whether *o,p'*-DDT acts through ERα and/or PXR, two reporter constructs were used: the CYP3A4-1200 reporter vector containing one PXRE and three ERE, and the p3A4-362(7836/7208 ins) reporter construct containing two PXRE and one ERE. Transactivation studies revealed that 10 μM *o,p'*-DDT treatment only mildly activated the CYP3A4-1200 construct, resulting in a maximum 1.22-fold induction over that observed in vehicle-treated cells. A 1.5-fold increase in induction was observed when cells were co-transfected with the PXR or ERα expression vectors. On the other hand, treatment with 10 μM *o,p'*-DDT induced CYP3A4-1200 activity as efficiently as a 10 nM estradiol. This result is in agreement with the fact that the hormone has 1,000 times greater affinity for ERα than the pesticide (Nelson, 1974).

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When cells were transfected with the p3A4-362(7836/7208 ins) reporter vector and treated with *o,p'*-DDT, dose-dependent promoter activation was observed; the maximum activation produced a 2.8-fold greater induction than in vehicle-treated cells. Co-transfection of the PXR expression vector increased the induction effects to 4-fold greater than controls, and resulted in a 16-fold increase in CYP3A4 mRNA levels. These results are in agreement with previous observations where *o,p´*-DDT increases CYP3A4 promoter activity by 6-fold (Lemaire et al., 2004). Although ERE and ER6 located at the proximal promoter play a role in CYP3A4 induction, our findings indicate that the *o,p'*-DDT exposure increases CYP3A4 mRNA levels mainly through the XREM region, probably via activation of PXRs. However, the XREM region is a complex array of transcription factor-binding sites that include a response element highly homologous to the recognition sequence for the orphan retinoic acid receptor-related receptor α-1 (RORα1), a site with identity to a binding motif recognized by hepatocyte nuclear factor-4 (HNF4), a putative binding site for CAAT/enhancer binding protein and two potential GRE half-sites (Goodwin et al., 1999). Therefore, the CYP3A4 promoter activation by *o,p'*-DDT through these transcription factors can not be ruled out. EMSA studies showed that treatment with 10 μM *o,p*-DDT increased protein-ER6 complex formation; no complex formation was observed with an ER6 mutant or unlabeled ER6 probes. Together these results suggest that the pesticide activates the PXRs and thereby increases binding to the ER6 response element. Interestingly, ER6-protein complex formation is greater when cells were treated with 10 μM of *o,p'*-DDT than with 50 μM of rifampicin. These data differed from the transactivation studies in which rifampicin had greater promoter activation potency than the pesticide. It was reported that regions of the PXR ligand binding domain (LBD) are mobile

and can change position when bound by distinctly shaped ligands (Orans et al., 2005). These ligand-dependent conformational changes may modify corepressor or coactivator binding, and these changes may underlie the discrepancy observed between the amount of ER6-protein complex formed and the transactivation response.

The promoter activation mediated by *o,p'*-DDT resulted in an increase in CYP3A4 activity measured by the cell capacity to metabolize testosterone. After 48 h treatment, a significant increase of 6β-, 16β-, 2α-testosterone and 6-dihydroxytestosterone were observed. Others CYPs may be involve on testosterone metabolism, particularly CYP2C9 and CYP2C19. It has been reported that hydroxylation activities at the 16β-position is higher in CYP2C9 and CYP2C19 than that of CYP3A4. However, 15β-, 6β-, and 2β-hydroxylation of testosterone are catalyzed most actively by CYP3A4 (Yamazaki et al., 1997). Since CYP2C9 and 2C19 genes are regulated by PXR, the contribution of these enzymes on testosterone 16β-hydroxylation has to be considered. Besides the potential to interact with androgen and estrogen receptors, these results clearly indicate that this pesticide has the capacity to alter testosterone levels by increasing the CYP3A4 activity and testosterone catabolism. Therefore, it is probable that levels of other endobiotics metabolized by this enzyme are also modified. Moreover, it was demonstrated in humans that exposure to DDT increase estradiol metabolism (Poland et al., 1970); (Nhachi and Loewenson, 1989). This is of particular interest since estradiol metabolites, such as 16-hydroxyestradiol, are estrogen receptor agonists associated with increased breast cancer incidence (Kabat et al., 1997). In accordance, a positive correlation between CYP3A4 activity and breast cancer has been also observed (Huang et al., 2003).

Species differences were found among the several animal models that have been proposed to assess xenobiotic effects on P450 regulation. Because CYP3A4 is expressed only in humans and few *in vivo* models are available, a transgenic mouse model expressing the human *CYP3A4* gene was used. Oral treatment with 1 mg/kg of *o,p'*-DDT increased CYP3A4 mRNA levels by over 30-fold relative to controls. Similar effects were observed in the experiments examining mouse Cyp3a11 mRNA levels. It is relevant to mention that adipose tissue samples from nonoccupationally exposed subjects were found to have a concentration of 1.79 mg/kg of *o,p'*-DDT and 17.45 mg/kg of total DDT (Waliszewski et al., 2004). Thus, modulation of *CYP3A4* gene transcription by this pesticide as observed in the mouse model may be occurring in human populations and could result in drug interactions and therapeutic failures or toxicities.

The levels of CYP3A4 induced by the pesticide treatment decreased by about 1/2 following 10 mg/kg *o,p'*-DDT treatment and was lost at 100 mg/kg. In humans, DDT is metabolized by CYP3A4 into the DDE and DDD metabolites (Kitamura et al, 2002). However, the sustained inductive capacity of the DDE metabolite was demonstrated in studies reporting that rats treated with DDE present an 11-fold increase in CYP3A1 mRNA levels and a 5-fold induction of a promoter activity driven by PXR response elements (Wyde et al., 2003). Although species differences on CYP3A regulation have been described, metabolic inactivation of *o,p'*-DDT seems not to be the respondible for the observed decrease in CYP3A4 induction.

It remains a possibility that the decreased CYP3A4 induction at the higher doses was due to an effect of *o,p'*-DDT treatments on PXR. It has long been known that hepatic metabolism is reduced during inflammation due to a cytokine-regulated decrease in P450 levels (Muntane-Relat et al., 1995). On the other hand, others found that *o,p'*-DDT induces

the production of several cytokines including IL-6 in macrophages (Kim et al., 2004). Moreover, IL-6 negatively regulates the expression of PXR and CAR as well as of CYP3A4 mRNA in primary human hepatocytes (Pascussi et al., 2000). In the present study, a moderate increase in IL-6 mRNA level was observed after *o,p'*-DDT treatment in the mouse intestine. These results explain, at least in part, the drop in CYP3A4 and Cyp3a11 induction produced by the pesticide. The mechanism by which this cytokine acts still unknown. However Jun/AP-1, a transcription factor up regulated by IL-6, was shown to interact and inhibit the transcriptional activity of RXRα, a heterodimerization partner of PXR (Zhou et al., 1999).

The sequence differences in the LBD of PXR account for most of the variations in CYP3A induction across species. Compounds that are know to differentially induce CYP3A expression in mouse, rat, rabbit or human activate only the corresponding PXR. For example, rifampicin activates human but not mouse PXR, while PCN is a weak activator of human and a much more potent activator of mouse PXR (Kocarek et al., 1995). Our results indicate that *o,p'*-DDT activates both receptors, suggesting that the differences in the LBD between human and mouse PXR do not affect the pesticide capacity to induce expression of CYP3A P450s. It is also noteworthy that is was demonstrated in transgenic mouse studies that the mouse PXR can activate CYP3A4.

In conclusion, the present study revealed that the pesticide *o,p'*-DDT induces the *CYP3A4* gene promoter through PXR, resulting in an increase in CYP3A4 mRNA and activity levels. We also showed that *o,p'*-DDT was able to increase CYP3A4 and Cyp3a11 mRNA levels, *in vivo*. Finally, *o,p'*-DDT may induce others genes that are under PXR regulation such other P450s (e.g. CYP3A7, CYP2C8, CYP2C9, CYP2C19 and CYP2B6),

phase II enzymes (UDP-glucoronosyltransferases and glutathione-S-transferases), efflux pumps (multidrug resistence proteins 1 and 2), and genes involved in bile acid metabolism and transport (Orans et al., 2005). Further experiments will be necessary to study these possibilities.

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Footnotes

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Figure legends

Fig. 1. Effects of *o,p'*-DDT on *CYP3A4* promoter activity. HepG2 cells, transfected with 4 μg of CYP3A4-1200 reporter construct or 100 ng of the control vector pGL3, were treated for 24 h with *o,p'*-DDT or rifampicine (RFP), and luciferase activity was measured and normalized for transfection efficiency. Mean luciferase activity values ± S.D. of triplicate determinations are shown. *p< 0.05 vs.control (C).

Fig. 2. Effects of PXR and ERα over-expression on *CYP3A4* promoter activity. HepG2 cells were co-transfected with CYP3A4-1200 reporter construct and PXR (columns 2, 3 and 4) or ERα (columns 5, 6 and 7) expression vectors, and treated for 24 with *o,p'*-DDT (columns 3 and 6), rifampicin (RFP) (column 4) or 17β-estradiol (column 7). luciferase activity was measured and normalized for transfection efficiency. Mean luciferase activity values ± S.D. of triplicate determinations are shown. *p< 0.05 vs.control (column 1).

Fig. 3. Effects of *o,p'*-DDT and PXR on p3A4-362(7836/7208 ins) activity. A) HepG2 cells, transfected with 4 μg of p3A4-362(7836/7208 ins) reporter construct were treated for 24 h with *o,p'*-DDT or rifampicine (RFP), and luciferase activity was measured and normalized for transfection efficiency. Mean luciferase activity values ± S.D. of triplicate determinations are shown. * p < 0.05 vs.control (C, vehicle-treated cells). B) HepG2 cells were co-transfected with 4 μg of p3A4-362(7836/7208 ins) reporter construct and 4 μg of PXR expression vector, and treated for 24 h with 10μM *o,p'*-DDT or 50 μM rifampicin (RFP). Luciferase activity was measured and normalized for transfection efficiency. Mean

luciferase activity values ± S.D. of triplicate determinations are shown. *p< 0.05 compared with control (C, PXR transfected cells treated with vehicle).

Fig. 4. Effects of *o,p'*-DDT on protein binding to the ER6 element. A) Nuclear extracts (NE, 20 μg) of HepG2 cells treated with 10 μM *o,p'*-DDT (lane 3), 50 μM rifampicine (RFP, lane 4) or vehicle (ethanol or DMSO, lanes 2 and 5), were incubated with radiolabeled ER6 response element probe (lanes 2-5) and analyzed by EMSA. B) Nuclear extracts (NE) from HepG2 cells treated with 10 μM of *o,p'*-DDT or vehicle (ethanol), were incubated with radiolabeled ER6 probe alone (lanes 2 and 3), ER6 probe plus 25-, 50- and 100-fold molar excess of unlabeled ER6 (ER6UL, lanes 4, 5 and 6), or ER6mut probe (5'-ATATGTTCTCAAAGGAGAACAGTGA-3', lane 7). Lane 1 is radiolabeled probe alone. The arrows identify the shifted bands and the unbound radiolabeled probe.

Fig. 5. Effects of *o,p'*-DDT treatment and PXR over-expression on CYP3A4 mRNA levels. HepG2 cells were transfected with 4 μg of PXR expression vector and treated with 10 μM of *o,p'*-DDT for 24 h. Relative expression of CYP3A4 mRNA was determined by rtPCR. All measurements were normalized to a calibrated standard sample. Data represent mean values ± S.D. from two independent experiments for triplicate. *p< 0.05 vs. control.

Fig. 6. Effects of *o,p'*-DDT on CYP3A4 and Cyp3a11 mRNA expression in duodenum mouse. Mice were treated with increasing doses of *o,p'*-DDT (1, 10 and 100 mg/kg), PCN (50 mg/kg) or corn oil (control). After treatment duodenal samples were removed and relative expression of CYP3A4 (A) and Cyp3a11 (B) mRNA levels were determined by

rtPCR. All measurements were normalized to a calibrator sample. Data represent mean values ± S.D. from 5 animals. *p< 0.05 vs. control.

Fig. 7. Effects of *o,p'*-DDT on IL-6 mRNA levels in mouse duodenum. Mice were treated with *o,p'*-DDT (1, 10 and 100 mg/kg) or vehicle (corn oil). After treatment duodenal samples were removed and relative expression of IL-6 mRNA was determined by RT-PCR. Relative intensity values were normalized using GADPH transcript. Data represent mean values ± S.D. from 5 animals. *p< 0.05 vs. control.

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Table 1. Sequences of primers and probes used for real time quantitative PCR

Gene name	Sequence (5'- 3')	Fragment length (bp)
CYP3A4	Foward: CCTGGTGCTCCTCTATCTATATGGA	75
	Reverse: GGTGTGGGCCCTGGAATT	
	Probe (FAM): CATTCACATGGACTTTTTAAG	
Cyp3a11	Forward : GCTCCTAGCAATCAGCTTGGT	83
	Reverse : GCCCAGGAATTCCCTGTTTCTTAAA	
	Probe (FAM): CTCTACCGATATGGGACTCG	

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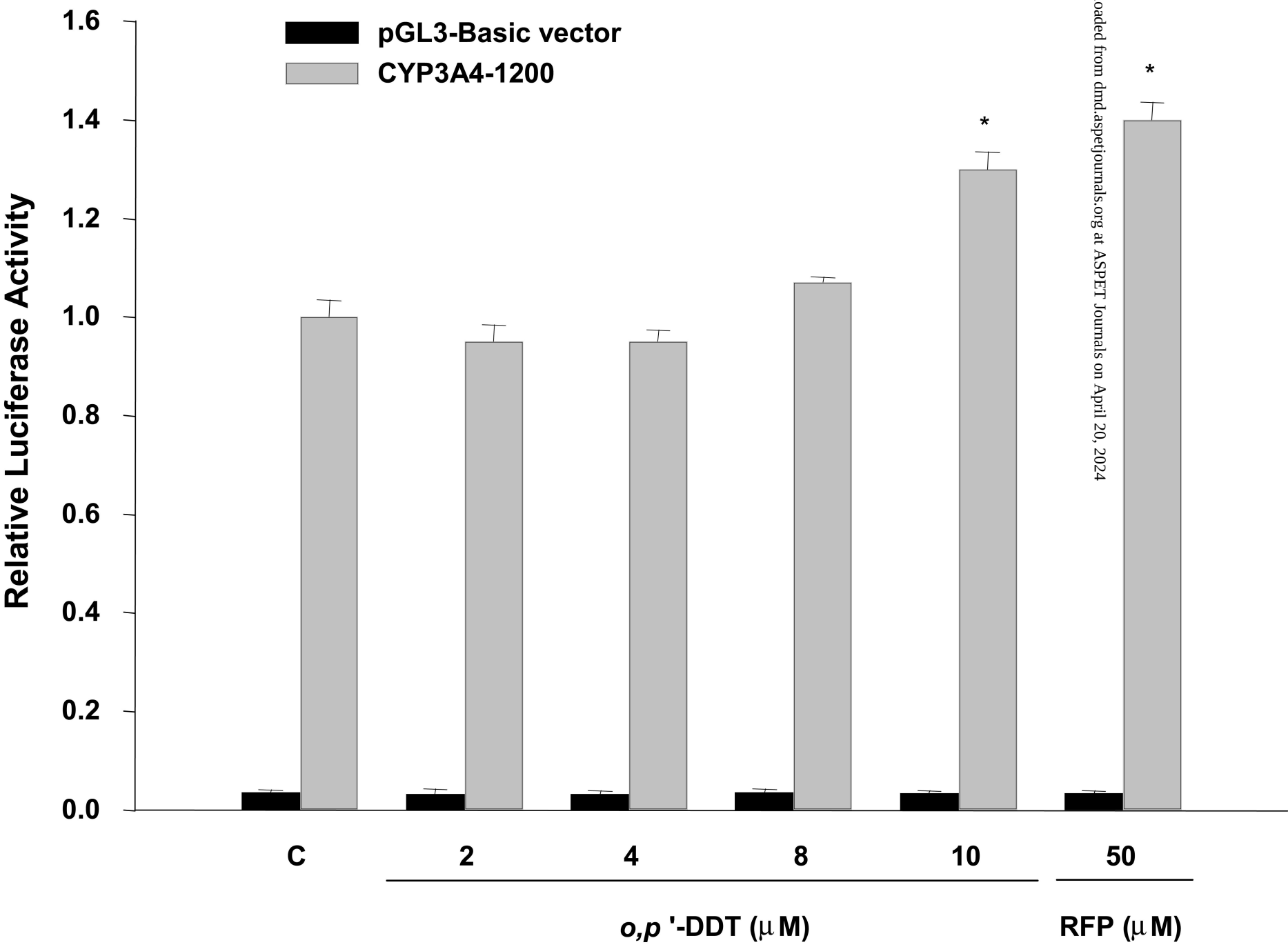
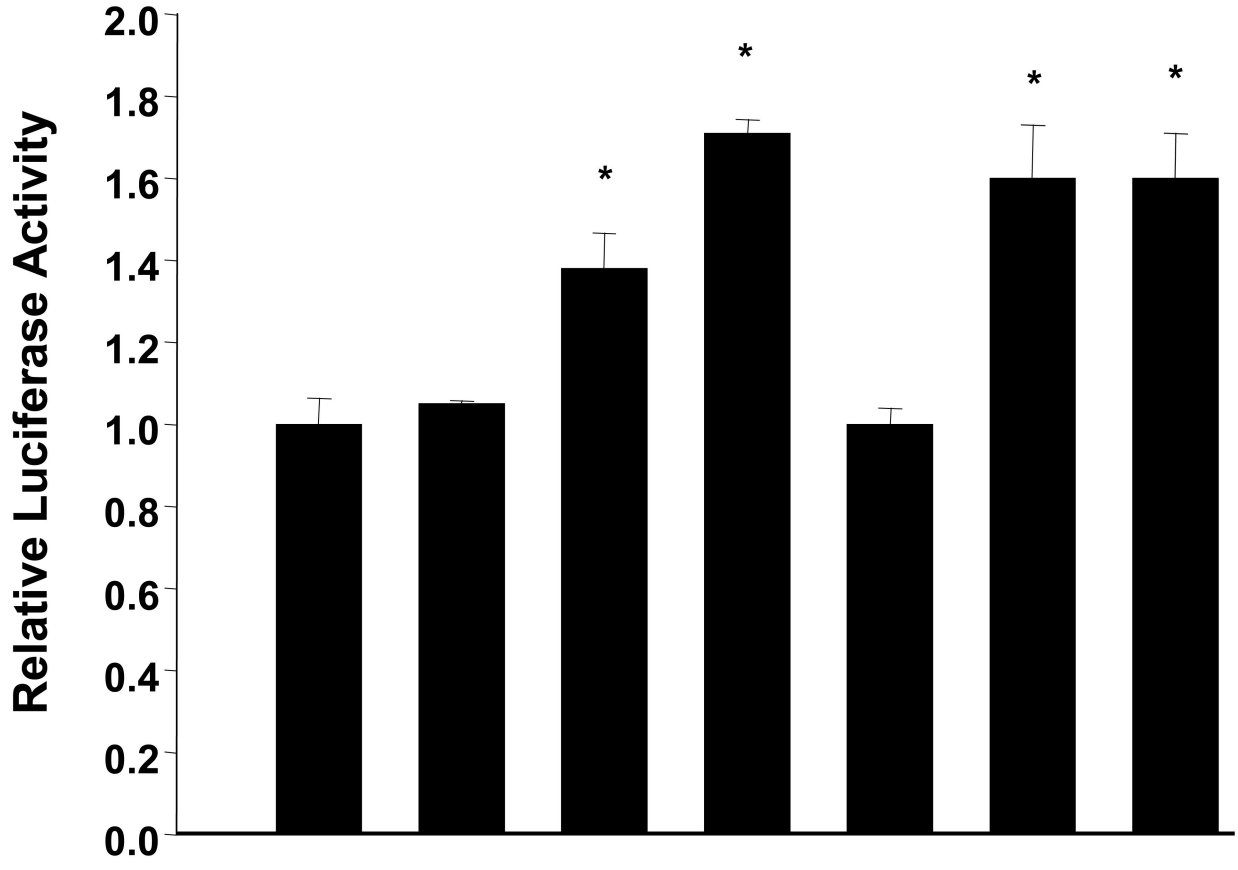
Table 2. Effects of *o,p'*-DDT on testosterone metabolism in HepG2 cells

	24 h treatment		48 h treatment	
Metabolites	Control	*o,p'*-DDT	Control	*o,p'*-DDT
15α-OHT	0.036±0.010	0.048±0.009	0.093±0.061	0.047±0.014
6-αOHT	0.093±0.017	0.093±0.002	0.098±0.010	0.109±0.004
6β-OHT	0.125±0.004	0.143±0.002*	0.154±0.015	0.191±0.017*
16β-OHT	0.056±0.009	0.051±0.008	0.065±0.008	0.097±0.013*
2α-OHT	0.089±0.005	0.098±0.005	0.122±0.014	0.173±0.027*
6-DHT	0.086±0.011	0.083±0.009	0.110±0.021	0.179±0.032*
Androstenadione	0.137±0.002	0.151±0.017*	0.201±0.037	0.243±0.033
NI-1	0.169±0.019	0.163±0.012	0.280±0.059	0.277±0.040
NI-2	0.025±0.003	0.036±0.004*	0.047±0.014	0.051±0.005

*15α-, 6α-, 6β-, 16β- and 2α-OHT, hydroxytestosterone, 6-DHT, 6-dihydrotestosterone, NI, non-identified. Data presented as nmol/mg protein, means ± S.D. (n=3). *p<0.05 vs non treated cultures.*

Figure 1





PXR (4 μg)	-	+	+	+	-	-	-
o,p'-DDT (10 μM)	-	-	+	-	-	+	-
RFP (50 μM)	-	-	-	+	-	-	-
ERα (4 μg)	-	-	-	-	+	+	+
17β-estradiol (10 nM)	-	-	-	-	-	-	+
Column	1	2	3	4	5	6	7

Figure 3A

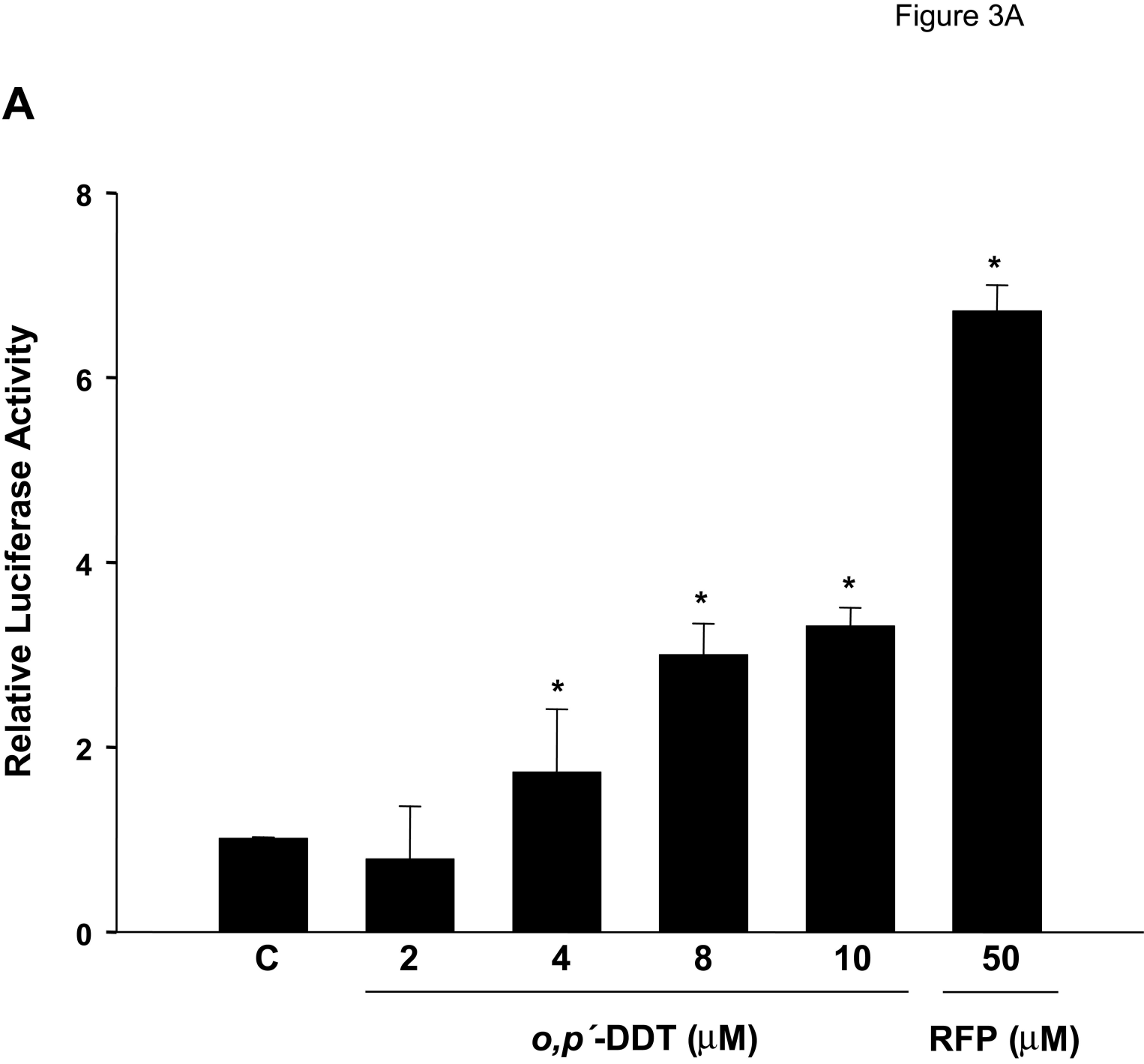
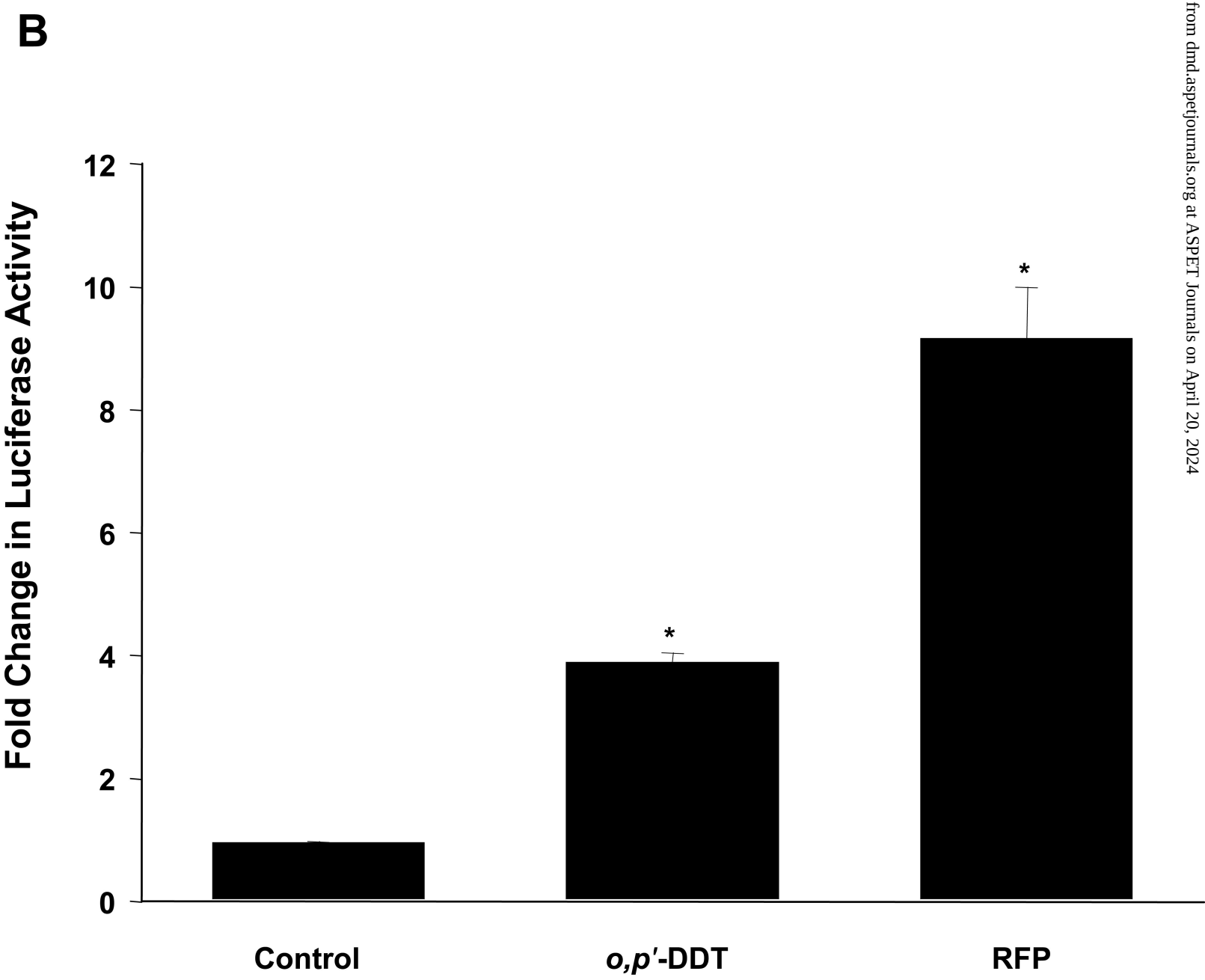
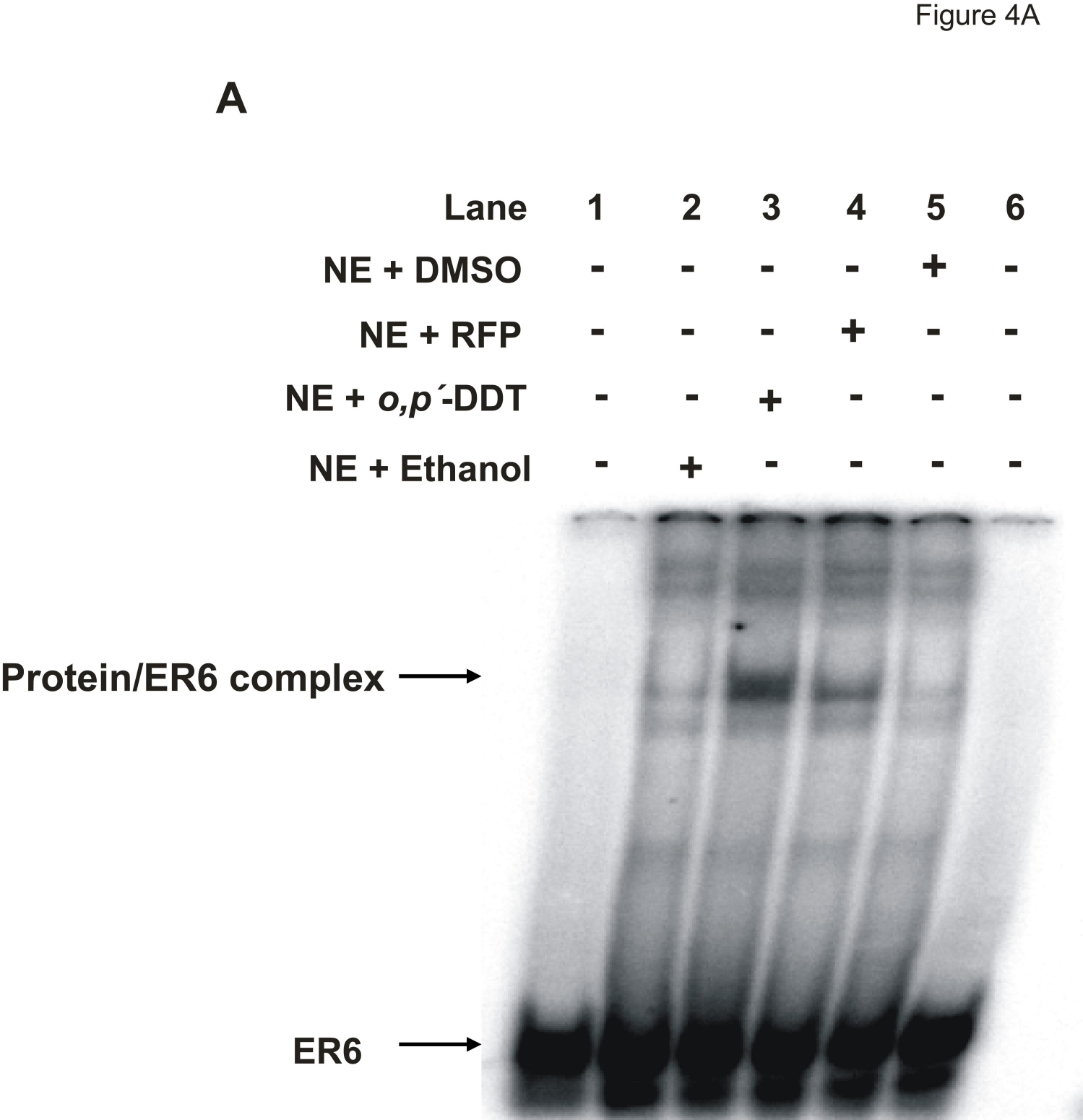

A
Relative Luciferase Activity
0
2
4
6
8
C
2
4
8
10
50
*
*
*
*
o,p´-DDT (μM)
RFP (μM)

Figure 3B




A
Lane 1 2 3 4 5 6
NE + DMSO - - - - + -
NE + RFP - - - + - -
NE + o,p´-DDT - - + - - -
NE + Ethanol - + - - - -
Protein/ER6 complex
ER6

B

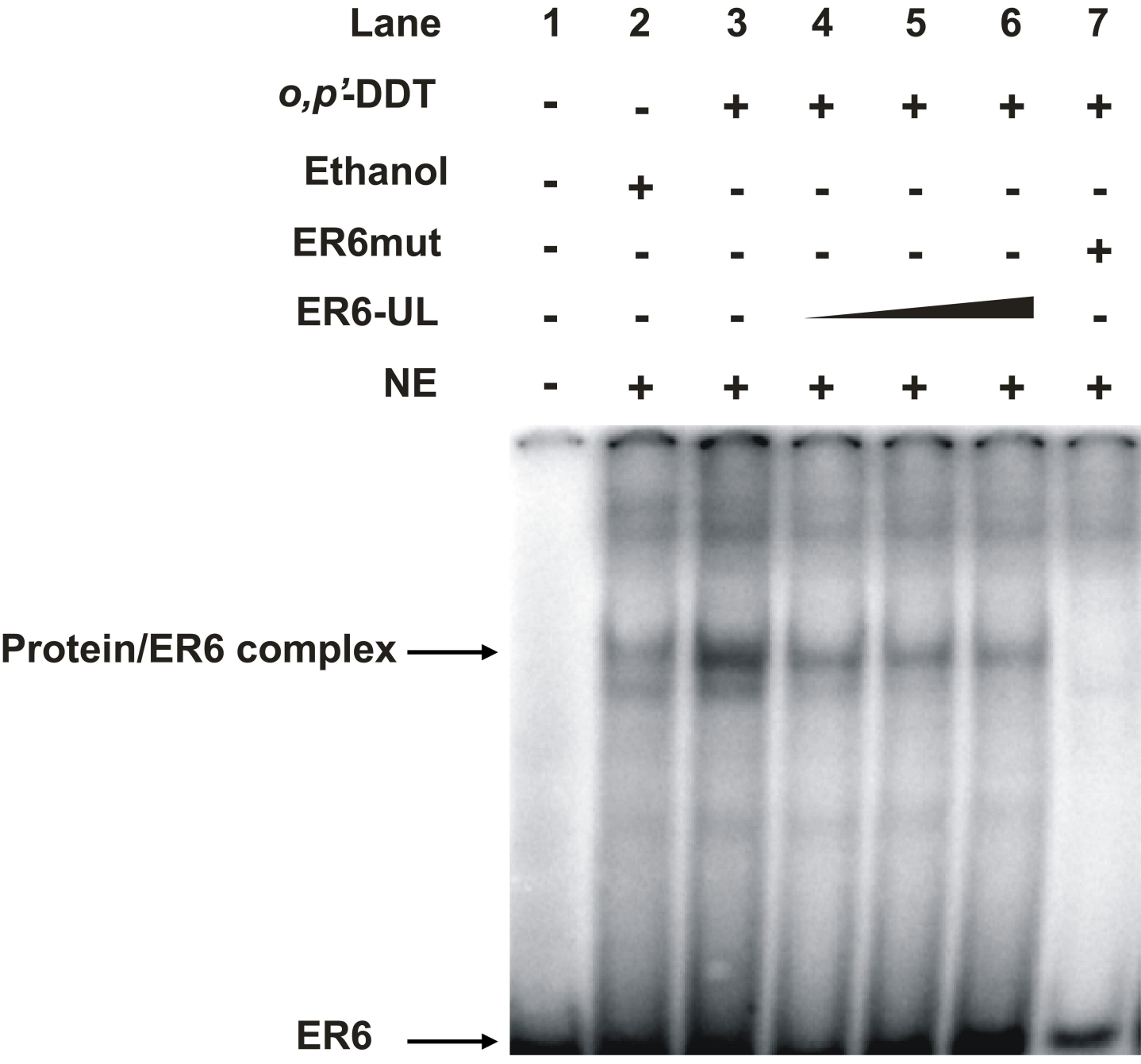

Lane 1 2 3 4 5 6 7
o,p'-DDT - - + + + + +
Ethanol - + - - - - -
ER6mut - - - - - - +
ER6-UL - - - - -
NE - + + + + + +
Protein/ER6 complex
ER6

Figure 5

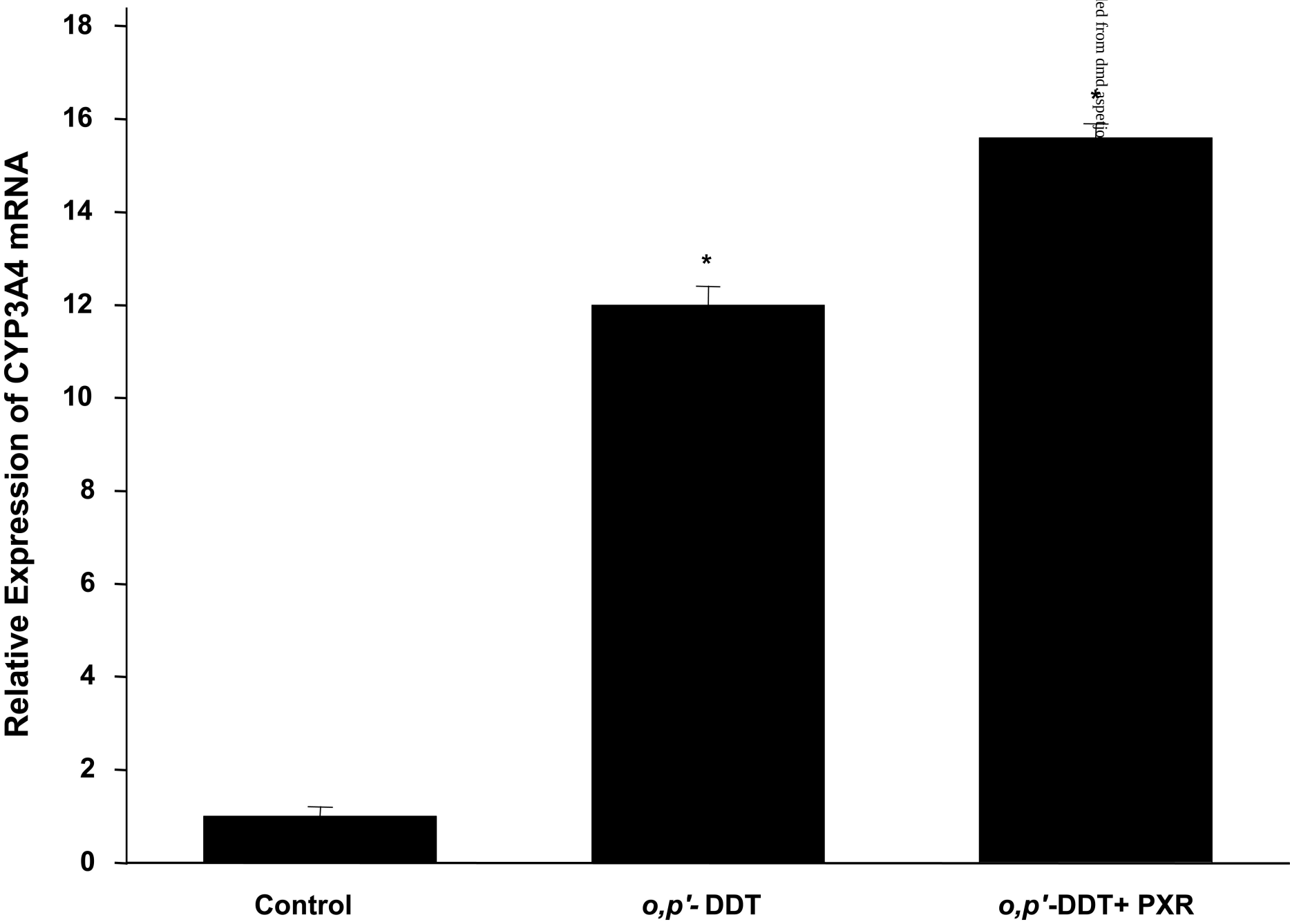


Figure 6A

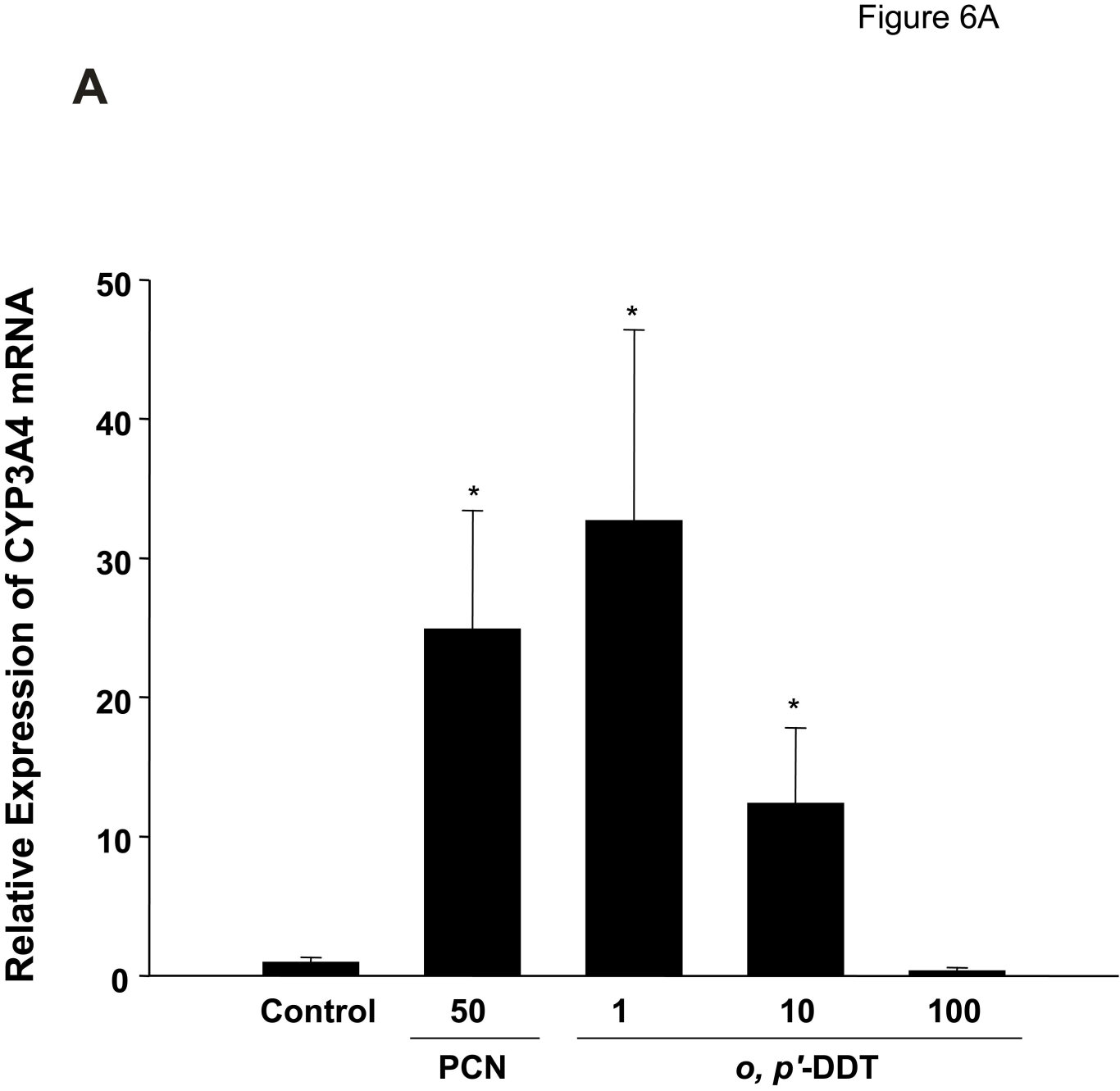

A
Relative Expression of CYP3A4 mRNA
50
40
30
20
10
0
*
*
*
Control
50
1
10
100
PCN
o, p'-DDT

Figure 6B

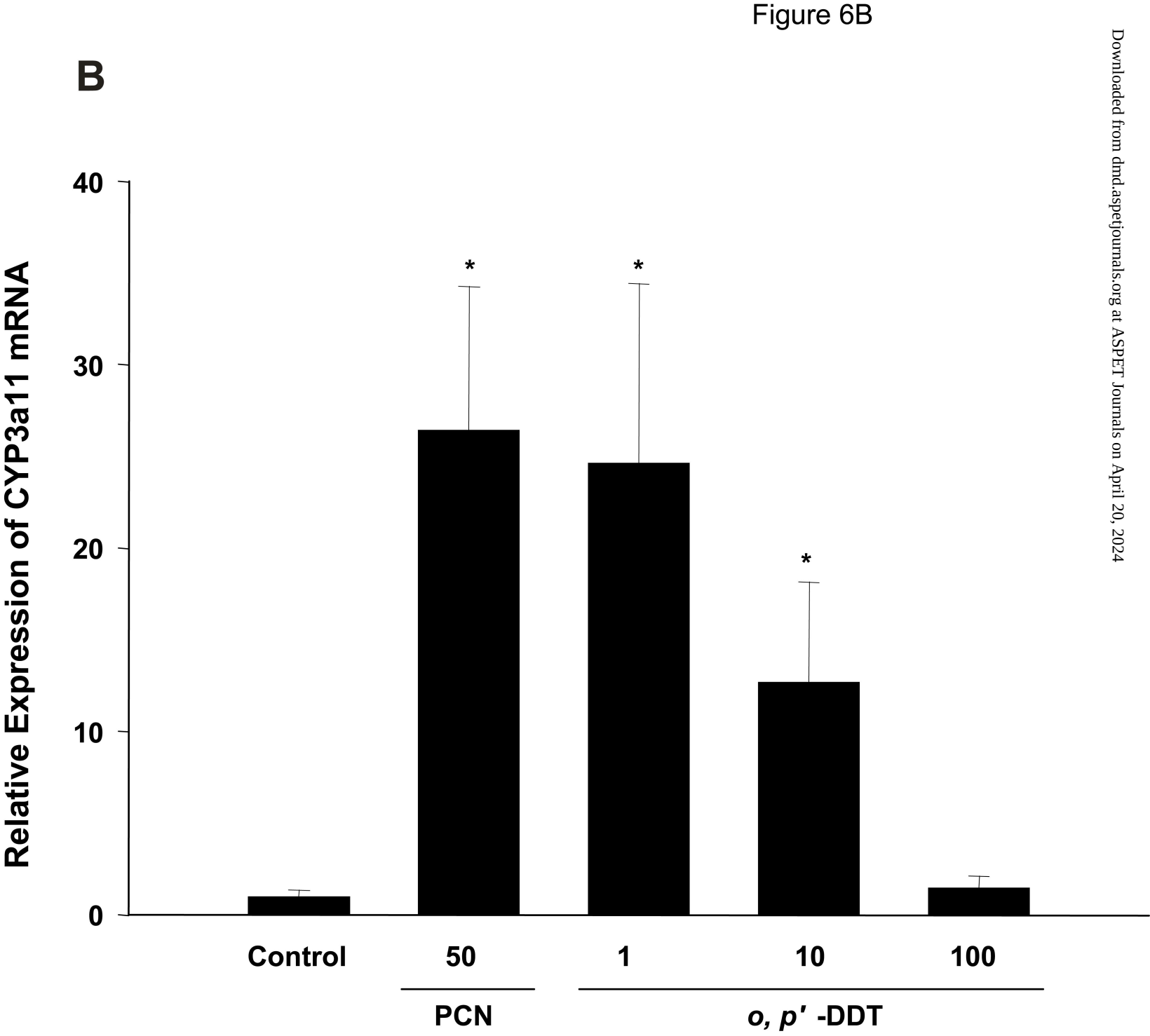
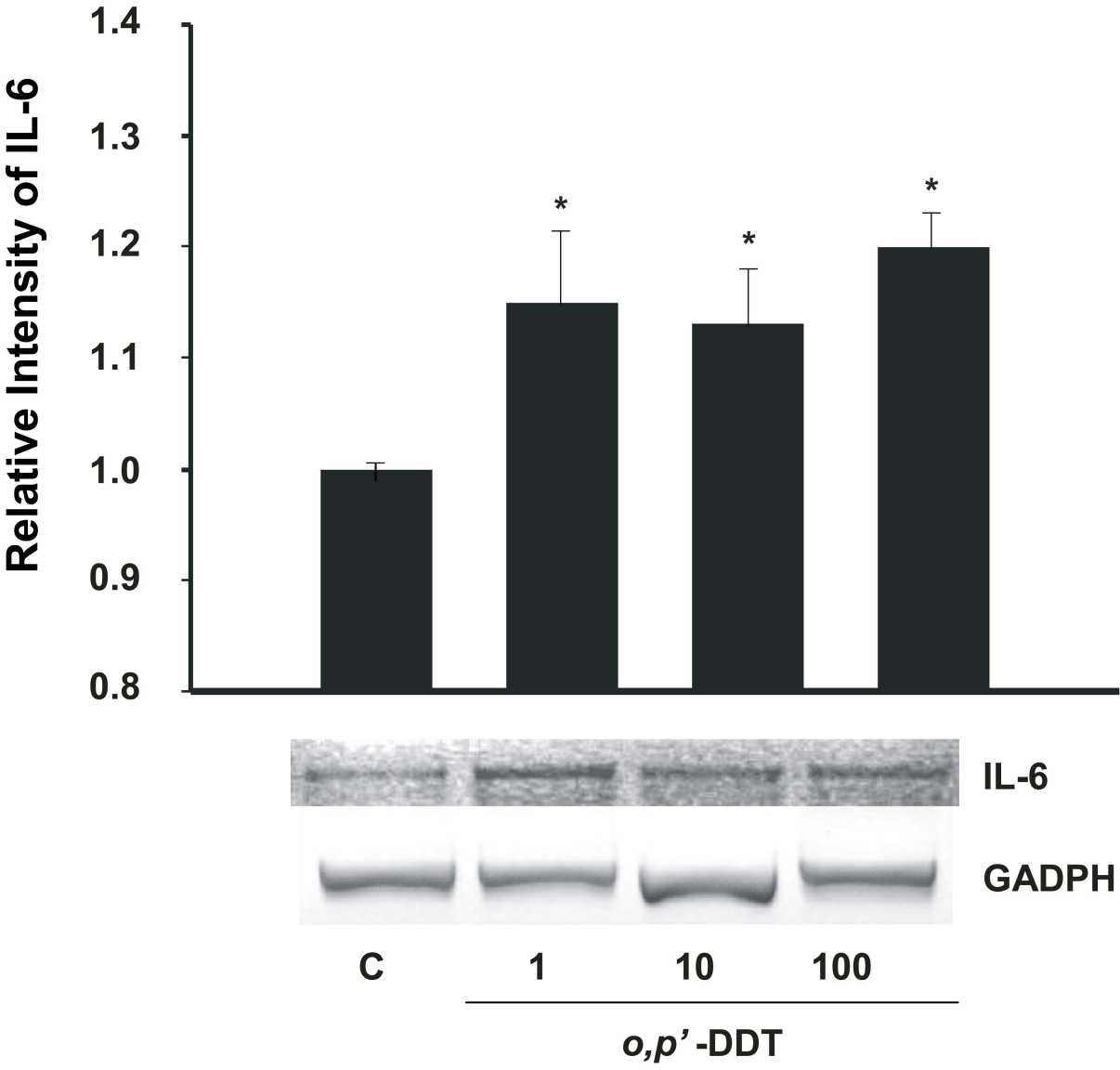


Figure 7


Relative Intensity of IL-6
1.4
1.3
1.2
1.1
1.0
0.9
0.8
*
*
*
IL-6
GADPH
C
1
10
100
o,p' -DDT