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Effects of triaryl phosphates on mouse and human nuclear receptors

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Abstract

The constitutively active receptor (CAR) is a crucial regulator of genes encoding for enzymes active in drug/steroid oxidation, conjugation, and transport. In our attempt to isolate the endogenous inhibitory ligand(s) for the mouse CAR, we found surprisingly that the inhibitory activity was associated with di- and tri-isopropylated phenyl phosphates that were present in livers of untreated mice. *Trans*-activation experiments in mammalian cells with synthetic compounds verified that mouse CAR was inhibited by various isopropylated phenyl phosphates (40–80%). Such triaryl phosphates are widely used as fire retardants, lubricants, and plasticizers, and some of them are known to disturb reproduction by currently unknown mechanisms. Equipped with the finding that these compounds could interact with mouse CAR, we proceeded to determine their functional effects on other nuclear receptors. Human CAR and pregnane X receptor (PXR) were variably activated (2–5-fold) by triaryl phosphates while mouse PXR, peroxisome proliferator-activated receptor-α, and vitamin D receptor were refractory. Among steroid hormone receptors, the human androgen receptor was inhibited by triphenyl phosphate and di-*ortho*-isopropylated phenyl phosphate (40–50%) and activated by di- and tri-*para*-substituted phenyl phosphates (2-fold). Our results add to the list of CAR and PXR activators and suggest steroid-dependent biological pathways that may contribute to the reproductive effects of triaryl phosphates.

Keywords: Organophosphates; Triaryl phosphates; Nuclear receptor; Activation; Mouse; Human

1. Introduction

NRs are ligand-dependent DNA-binding transcription factors that are encoded by a superfamily of 48 genes [1]. The NRs transmit extra- or intracellular signals directly to gene transcription machinery, in response to steroid and thyroid hormones, fatty acid and cholesterol derivatives, and vitamins A and D. NRs control profound cellular processes such as growth and differentiation, carbohydrate and lipid metabolism, and endocrine physiology [2]. Therefore, disturbances in function of NRs may result in clinical manifestations. Such disturbances include hormone resistance syndromes and cancers where interindividual differences in NR structure result in abnormal ligand binding or changes in responses to NR co-regulators [3,4]. On the other hand, environmental or diet-derived chemicals (*xenobiotics*) may disturb hormonal balance via binding to NRs [5–7]. Xenobiotics may also affect hormonal balance through activation of *CYP* gene expression. CYPs are essential not only in the metabolism of xenobiotics but also in the control of both formation and degradation of endogenous compounds (*endobiotics*) which include NR ligands [8]. Because *CYP* genes are regulated by NRs [9], exposure to xenobiotics may lead to increased expression of CYP enzymes and enhanced xeno- and endobiotic metabolism.

While the ligand specificities of steroid hormone receptors have been well established, many of the so-called

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Abbreviations: AR, androgen receptor; CAR, constitutively active receptor; CYP, cytochrome P450; ERα, estrogen receptor-α; GR, glucocorticoid receptor; NR, nuclear receptor; PPARα, peroxisome proliferator-activated receptor-α; PR, progesterone receptor; PXR, pregnane X receptor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; TPP, triphenyl phosphate; TTP, tri-*p*-methyl phenyl phosphate. Isopropylated triaryl phosphates are abbreviated by the generic symbol *o*-, *m*-, *p*-iPrXN, where *o*-, *m*-, and *p*- denote the position of the isopropyl (iPr) substituent on the phenyl moiety, and *XN* denotes the number of isopropylated phenyl moieties (N = 1, 2, or 3).

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orphan receptors lacked known ligands at the time of their discovery [10]. Ligands that were later identified for these orphan receptors by screening of chemical libraries may not be the actual effectors *in vivo*. For instance, farnesoid X receptor was originally reported to be activated by farnesol metabolites [11] but its true physiological ligands seem to be bile acids [12]. Similarly, retinoid X receptor binds 9-*cis*-retinoic acid with high affinity [13]; however, docosahexaenoic acid may be an important ligand *in vivo* [14]. Androstane metabolites that were identified as inverse agonists of the CAR [15] are present in blood at much lower levels than required for efficient deactivation of CAR [16]. In addition, significant species-specific ligand preferences for CAR and PXR that result from their divergent ligand binding domains complicate the understanding of the ligand specificity [9,17], in contrast with the highly conserved classical steroid hormone receptors.

Recent studies have established that CAR plays a critical role in expression of cytochrome P450 CYP2B enzymes in the liver [9,18]. CAR is a unique NR because its ligands are either activating or suppressing [17]. Because the main CAR target gene, *Cyp2b10*, is expressed at very low levels while CAR is constitutively active [19], there must be mechanisms to suppress CAR when inducers are not present. One such mechanism is the cytoplasmic location of CAR [20] that would keep the *Cyp2b10* gene in the basal state. However, this mechanism does not preclude the existence of endogenous CAR-suppressing ligands that may be required to keep CAR sequestered in the cytoplasm. Also, these ligands would not necessarily be identical to androstenol, the inverse agonist of CAR identified by chemical screening. Therefore, we attempted to purify ligands that would inhibit the activity of CAR from untreated liver tissue using extraction and high-performance liquid chromatography techniques coupled with cell-based, CAR-responsive reporter gene system and mass spectrometry. Surprisingly, CAR-suppressing activity was associated with man-made chemicals, isopropylated TPPs. Experiments with synthetic compounds verified their suppression of CAR activity. In contrast to suppression of mouse CAR, triaryl phosphates had activating effects on human CAR and PXRs. Certain triaryl phosphates proved to be partial suppressors of the human AR while their effects on other steroid hormone receptors were marginal. Our results suggest potential pathways by which triaryl phosphates may exert some of their biological effects.

2. Materials and methods

2.1. Chemicals

The synthesis of TCPOBOP has been described [21]. Steroids and other chemicals were either from Steraloids Inc. or Sigma–Aldrich except that testosterone was from Makor Chemicals. The reagents and solvents for synthesis (reagent or HPLC grade) were purchased from Sigma–Aldrich.

2.2. Synthesis and purification of triaryl phosphates

TTP and tri-*o*-, tri-*m*-, and tri-*p*-isopropyl phenyl phosphates (*o*-, *m*-, *p*-iPrX3) were prepared from phosphorus oxychloride, di-*o*-, di-*m*-, and di-*p*-isopropyl phenyl phosphates (*o*-, *m*-, *p*-iPrX2) from phenyl dichlorophosphate, and *o*-, *m*-, and *p*-isopropyl phenyl phosphates (*o*-, *m*-, *p*-iPrX1) from diphenyl chlorophosphate and the appropriate phenol [22]. All trialkylated and *o*- or *m*-substituted compounds were purified on silica gel using hexane/ethyl acetate (8:2) as eluent. Other compounds were recrystallized from ether. All purified compounds were more than 99% pure, as judged by ^{1}H- and ^{13}P-NMR spectroscopy, except the *o*-substituted compounds for which the purity was 97% (2-alkylphenol as the impurity). TPP (99% pure) was a kind gift from Prof. Conny Östman (Department of Analytical Chemistry, University of Stockholm).

2.3. Purification of mCAR-deactivating fractions from liver tissue

The use of plastic ware was avoided throughout the procedure. Livers of untreated male Balb/c//Kuo mice were perfused *in situ* with ice-cold saline and snap-frozen in liquid N_2. Liver tissue was homogenized in equal volume of water on ice bath, and briefly treated with 1 M KOH at 70°. The homogenate was extracted three times with diethyl ether, the organic phase was dried with anhydrous Na_2SO_4, and the solvent was evaporated. The residue was dissolved in a minimal volume of dichloromethane and chromatographed on activated silica gel column (10 cm × 2 cm; Kieselgel 60; Merck) with methanol/dichloromethane (1:9). In this and subsequent steps, aliquots from fractions were evaporated to dryness and redissolved in isopropanol. A portion (1.5–10% of the original fraction) was tested for mouse CAR deactivation as described below. Fractions displaying specific deactivation of mouse CAR-mediated luciferase activity were pooled. The pooled fractions from the silica gel step were concentrated and chromatographed at 1 mL/min on Hibar silica gel columns (25 cm × 0.46 cm; Merck) with a gradient that consisted of hexane/dichloromethane (1:1; A) and dichloromethane/ethyl acetate (6:4; B) (10 min 100% A, 10–30 min 100%A–50%A, 30–40 min 50%A–0%A, 40–50 min 0%A). One milliliter fractions were collected, and mouse CAR-inhibiting fractions were pooled (Pool 1: fractions 7–12; Pool 2: fractions 24–30). These pools were then fractionated on sequential reversed-phased chromatography on Zorbax ODS (25 cm × 0.46 cm, DuPont; 28–100% ACN with 0.01% trifluoroacetic acid over 0–60 min) and Purospher RP-18 endcapped columns (12.5 cm × 0.4 cm; Merck; 40–100% ACN with 0.01% trifluoroacetic acid over 0–90 min).

2.4. Identification of the compounds in active fractions

The purified fractions were first analyzed by gas chromatography-mass spectrometry (GC–MS; Trio-2 quadrupole spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph, VG Masslab). One microliter samples were introduced to GC by splitless injection (splitless time 30 s, injector at 250°). The compounds were separated on a Hewlett-Packard methylsilicone capillary column (HP-1, 12 m × 0.2 mm, film thickness 0.33 μm) with a temperature gradient (2 min at 120°, followed by 180–320° over 20 min). The molecules were ionized by 36 eV electrons and the mass range *m/z* 50–700 was scanned in 0.9 s. The compounds were identified by comparisons with the data in Wiley mass spectral library. The identification of the compounds was verified by running a commercial tri-(isopropylphenyl) phosphate standard (Chem Service Inc.) with the GC–MS instrument. The elemental composition of selected compounds was then determined with a high resolution GC–MS instrument (VG250, 70-SE) with above GC conditions. The accurate mass measurement was carried out automatically with the data system. Perfluorokerosene was used as the reference compound.

2.5. Reporter plasmids

pCMVβ was purchased from Clontech Inc. pARE$_2$-TATA-LUC reporter contains two AR response elements in front of minimal TATA sequence [23]. Hormone response elements in the ARE$_2$TATA promoter mediate also GR- and PR-dependent signaling [24]. pERE$_2$TATA-LUC was constructed in the same way except that the inserted 45-base pair oligomer contained two ER response elements [25]. The reporter plasmids pPBREM-tk-luc [26], pXREM-3A4-luc [27] and pDR1x4-tk-luc [21] which respond to CAR, PXR, and PPARα, respectively, have been described.

2.6. Expression plasmids

The sources of expression vectors for mouse and human PPARα, PXR, and CAR, and for human vitamin D receptor have been described [21]. Human AR, GR and ERα expression vectors have been reported earlier [23,25,28]. pSG5-hPR1 was gift from Dr. Pierre Chambon (INSERM, Illkirch, France). All plasmids were purified with Qiagen columns.

2.7. Cell culture and transfection with mouse and human CAR, PXR, and PPARα

HEK293 cells (ATCC CRL-1573) were grown in phenol-free Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. One day prior to transfection, 70 × 10^3 cells per well were seeded on 48-well plates in medium containing 5% delipidated FBS (Sigma). After an overnight incubation, the medium was changed and the cells were transfected using calcium phosphate method. In the mouse CAR deactivation assay, each well received pCMVβ (100 ng), UAS$_4$-tk-luc (25 ng) [29] and CMV-GAL4-mouse CAR (12.5 ng) [21]. In experiments with purified test chemicals, each well received pCMVβ (100 ng), expression vectors (12.5 ng) for full-length mouse or human CAR, PXR, or PPARα receptors, and the respective reporter gene (25 ng). After a 4-hr transfection period, the medium was changed. The fresh medium additionally contained either samples from HPLC fractions that were first evaporated and then dissolved in isopropanol (mouse CAR assay), or purified test chemicals. Controls for mouse CAR included 10 μM androstenol (suppressor) and 0.5 μM TCPOBOP (activator) [30]. To account for the known species differences in activation of xenobiotic-activated NRs [17], positive controls for human CAR, mouse PXR, human PXR and both PPARα species were 2 μM clotrimazole, 10 μM RU486, 10 μM rifampicin and 10 μM Wy-14,643, respectively [21]. Cells were then cultured for 40 hr prior to washing with PBS and lysis of the cells. Luciferase and β-galactosidase activities [26] were determined from 20 μL of lysates in 96-well plates using the Victor^{2TM} multiplate reader (Perkin-Elmer Wallac).

2.8. Cell culture and transfections with human GR, PR, AR, and ERα

COS-1 cells (ATCC CRL-1650) were maintained in Dulbecco's minimal essential medium containing antibiotics and 10% FBS. Cells were seeded onto 12-well plates and transfected 24 hr later by FuGene transfection method (Roche Molecular Biochemicals). In brief, 30 × 10^3 cells in each well received 290 ng of the luciferase reporter plasmid, 20 ng of pCMVβ internal control plasmid and 20 ng of different steroid receptor expression vectors. Four hours before transfection, the medium was changed to one containing 10% charcoal-stripped FBS. Twenty hours after transfection, the cells received fresh medium containing 2% charcoal-stripped FBS with or without indicated steroid agonist (10 nM) or test compound. Forty-eight hours after transfection, the cells were harvested, lysed in Reporter Lysis Buffer (Promega), and the cleared supernatants were used for luciferase measurements with reagents from Promega using a Luminoskan Ascent luminometer (Labsystems) and for β-galactosidase assays as described [31,32].

2.9. Statistical methods

After normalization for transfection efficiency using β-galactosidase activity, reporter gene activities are expressed relative to those of vehicle (set at unity). Analysis of variance was employed. *P*-values <0.05 as compared to vehicle were considered statistically significant.

3. Results

Fractions that contained substance(s) inhibitory to mouse CAR were first enriched by extraction and step-wise elution from silica gel, and then purified by silica gel HPLC. From this step, two pools were further separated on a Purospher RP-18 column (Fig. 1, upper panels) and tested for mouse CAR inhibition (Fig. 1, bottom panels). The positive control TCPOBOP activated mouse CAR 2.6–2.9-fold and the negative control androstenol inhibited the activity by 80–90%, as expected [20,29]. From Pool 1, fractions 35–37 and 49–50 inhibited mouse CAR by about 60%. From Pool 2, fractions associated with peaks eluting at 37.8 and 39.5 min (fractions 38 and 40) inhibited mouse CAR very potently, almost reaching the androstenol-suppressed levels. Minor inhibiting activities were also found in fractions 27, 41, and 52–53 (51–63% of activity remaining). No inhibitory fractions were found when liver tissue was omitted from the purification scheme (data not shown), indicating that the active compounds were not contaminants from the purification but originated from the liver.

The active fractions were analyzed with gas chromatography and electron ionization mass techniques (Fig. 2). From Pool 1, fractions 35–37 yielded a major molecular ion of 368 Da (Fig. 2A) and fractions 49–50 gave an ion of 410 Da (data not shown). From Pool 2, major molecular ions from fractions 27, 38–42 + 44, and 52–53 had masses of 408/410, 450/452, and 492 Da, respectively (Fig. 2B–D). All these ions exhibited a common fragmentation pattern: neutral loss of an isopropylphenyl moiety (M-117), an

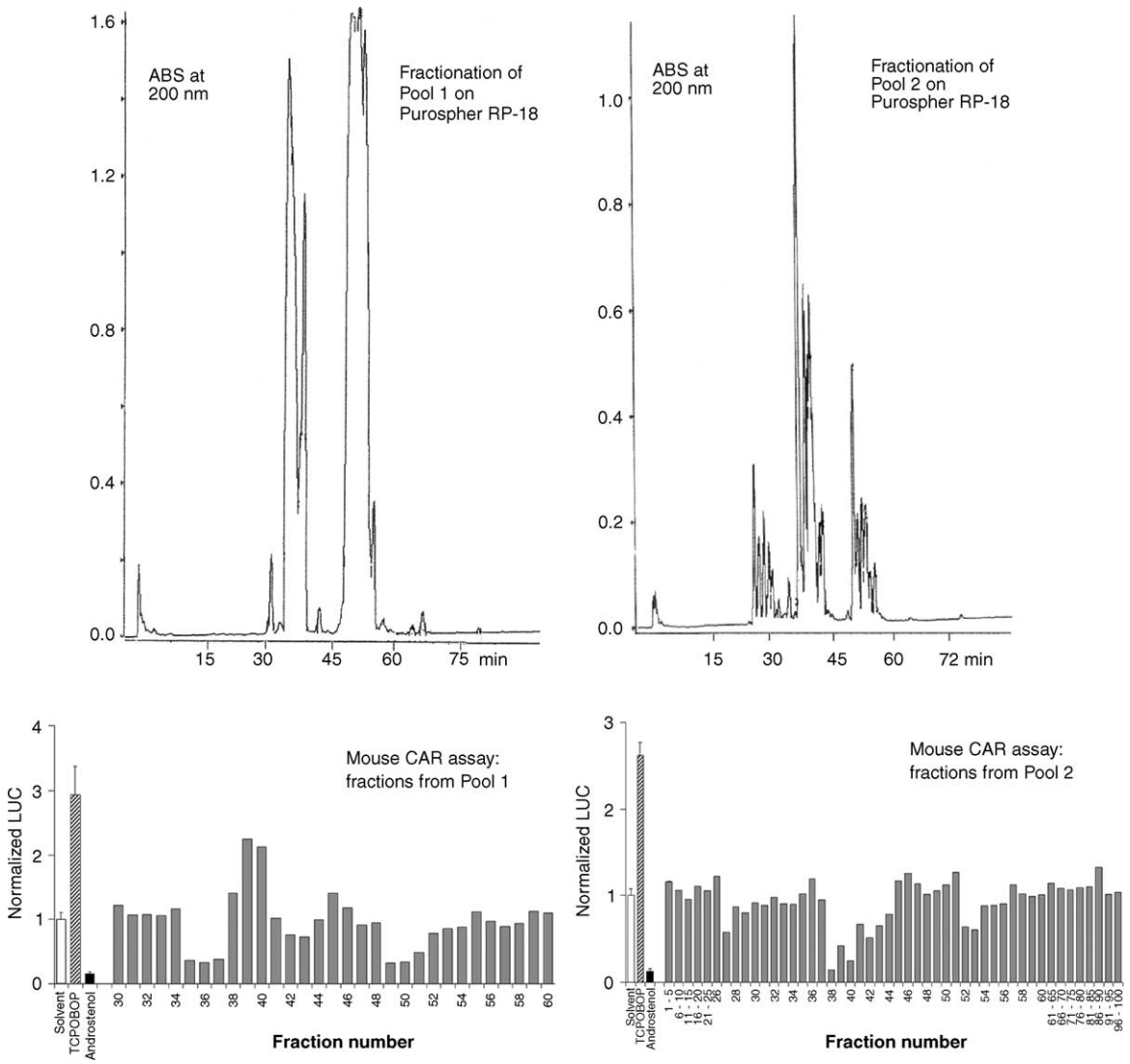
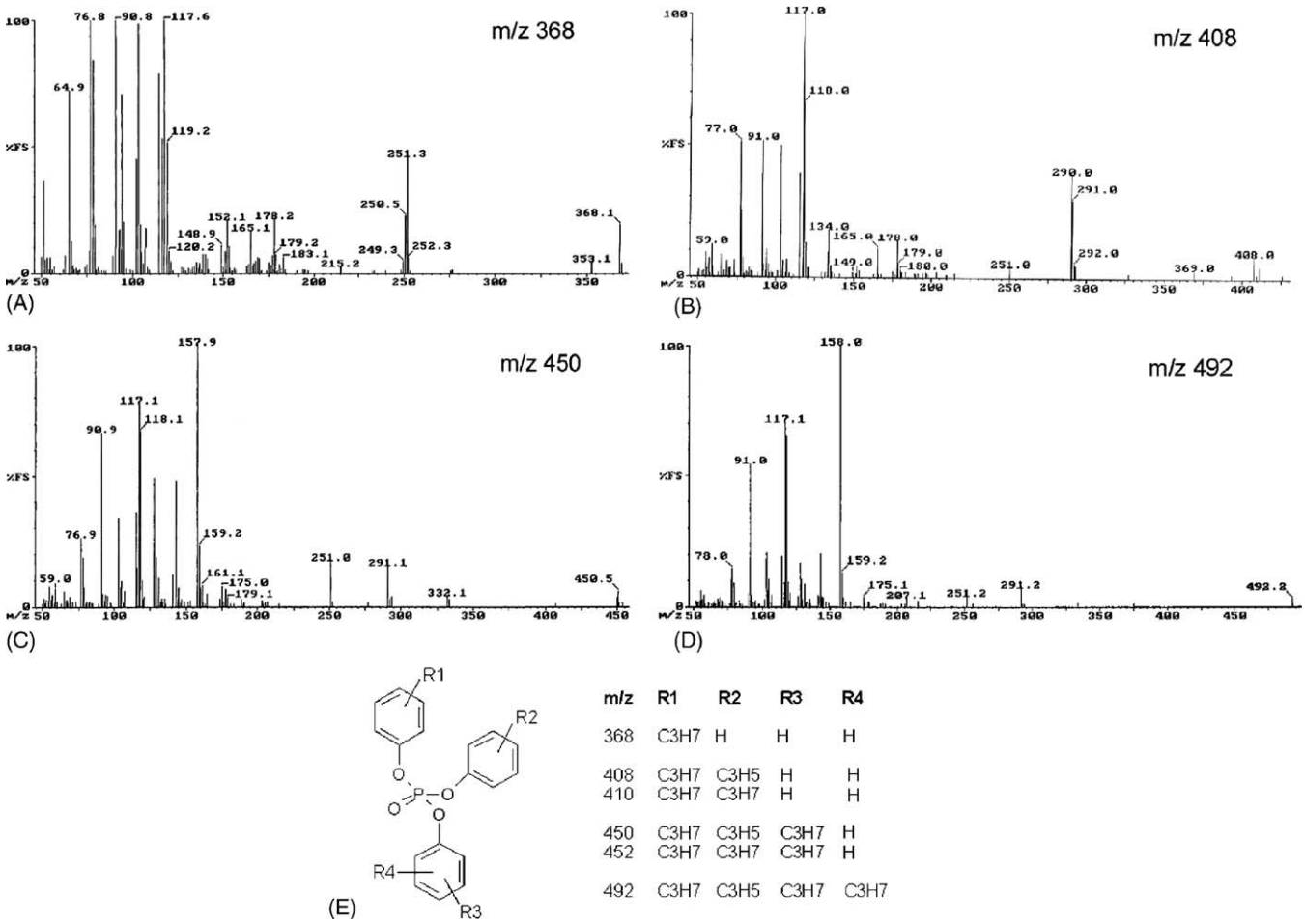


Fig. 1. The separation of liver-derived substances inhibitory to mouse CAR. Mouse liver was extracted and chromatographed on silica gel and Zorbax ODS reversed phase columns. The fractionations of silica gel Pool 1 (upper left) and Pool 2 (upper right) on Purospher RP-18 column are shown. The column effluent was monitored at 200 nm. Aliquots (3% for Pool 1; 1.5% for Pool 2) were tested for effects on mouse CAR activity (bottom panels) as described in Section 2. Solvent (set to 100; empty), 0.5 μM TCPOBOP (hatched), and 10 μM androstenol (filled) are shown as references. In Pool 1, fractions 35–37 and 49–50 displayed significant inhibition of mouse CAR (bottom left). In Pool 2, peaks eluting at 37.8, 39.5, and 41.1 min had the strongest inhibition while significant inhibition was also observed with peaks eluting at 26.6, 51.6, and 52.8 min.



m/z	R1	R2	R3	R4
368	C3H7	H	H	H
408	C3H7	C3H5	H	H
410	C3H7	C3H7	H	H
450	C3H7	C3H5	C3H7	H
452	C3H7	C3H7	C3H7	H
492	C3H7	C3H5	C3H7	C3H7

Fig. 2. The mass spectra of substances detected in active fractions. The molecular ions and the fragments thereof are shown for Pool 1: fraction 35 (A), Pool 2: fractions 27 (B), 38 (C), and 52 (D). The basic structure of triaryl phosphates (E) is shown, with substituents (R1–R4) consistent with data from mass spectra A–D. Please note that the single C_3H_5 group detected can be any of the R1–R4 groups.

isopropylphenyl ion at *m/z* 118 and ions at *m/z* 77 and 91, which are typical for phenylalkyl compounds [22]. The fragmentation spectrum of the 408 Da ion corresponded with that of mono-(*ortho*-isopropylphenyl)-mono-(*ortho*-isopropenylphenyl) phenyl phosphate in the Wiley mass spectral library. The molecular formula of the *m/z* 408 ion was verified with high-resolution gas chromatography/mass spectrometry. The measured molecular weight was 408.152631 Da, with only a 3.6 mDa difference to the calculated value of 408.149048 Da for $C_{24}H_{25}O_4P$. The structures of the other compounds in the molecule series could be explained by addition of different number of isopropyl (C_3H_7; 43 Da) and isopropenyl (C_3H_5; 41 Da) side chains to TPP backbone (Fig. 2E). In summary, the active fractions contained TPPs with isopropyl (N = 1–4) and isopropenyl groups (N = 0 or 1).

To verify the effects of triaryl phosphates on mouse CAR, several isomers of mono-, di- and tri-isopropylphenyl phosphates were then synthesized. Due to the small amount of the di-(*ortho*-isopropylphenyl)-mono-(*ortho*-isopropenyl-phenyl) phosphate available, extensive biological tests with this compound were not feasible. Figure 3 shows the responses of mouse and human CAR and PXR receptors to various triaryl phosphates (10 μM) and the reference compounds. As expected, mouse CAR was activated by TCPOBOP and inhibited by >90% by androstenol. TTP activated mouse CAR by 2-fold while the response to TPP was weaker (1.3-fold). Isopropylated phenyl phosphates gave a complex pattern with mouse CAR. *Ortho*-substituted compounds decreased the activity by 40–80% ($P < 0.05$), with higher degree of substitution correlating with the degree of inhibition. Increasing the number of *meta*-substituted groups changed the response from slight inhibition (*m*-iPrX1) to no effects (*m*-iPrX2, *m*-iPrX3). In addition, the 2–2.5-fold activation by mono- and di-*para*-substituted compounds was lost when the third isopropyl group was added (*p*-iPrX3). Finally, the di-(*ortho*-isopropylphenyl)-mono-(*ortho*-isopropenylphenyl) phosphate that was detected in purified fractions also inhibited mouse CAR to similar extent (data not shown). These changes were not due to non-specific effects since the luciferase activity from a reporter gene lacking NR response elements or β-galactosidase activity that was used for reporter normalization were not changed by the

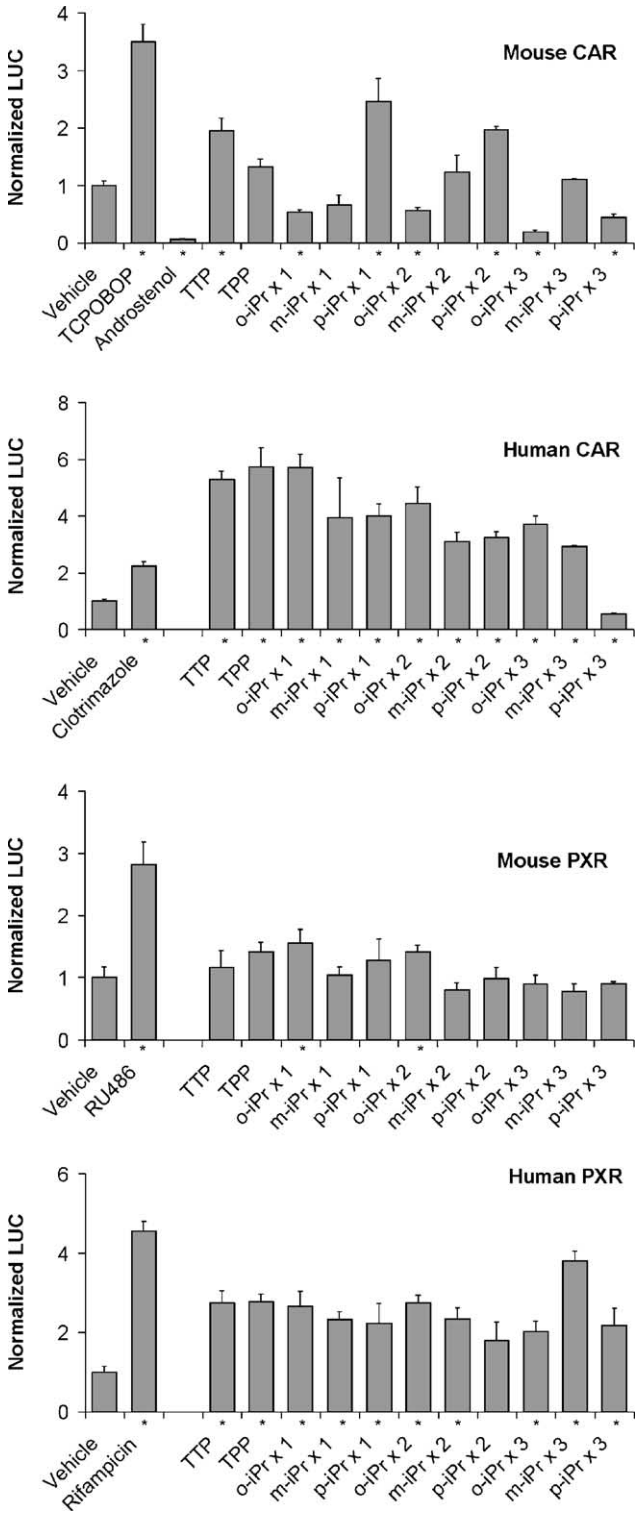


Fig. 3. The effects of triaryl phosphates on mouse and human CAR and PXR. HEK293 cells were transfected with indicated NRs and their reporter genes and treated with vehicle, reference substances (mouse CAR: 0.5 μM TCPOBOP and 10 μM androstenol; human CAR: 2 μM clotrimazole; mouse PXR: 10 μM RU486; human PXR: 10 μM rifampicin) or triaryl phosphates (10 μM). The data shown are means ± SD from three independent experiments, and expressed relative to vehicle (set to unity). The asterisks (*) below the columns indicate statistically significant differences in comparison with vehicle ($P < 0.05$).

compounds (data not shown). No overt toxicity was evident after the exposure of the cells to 10 μM triaryl phosphates. To summarize, these data confirmed that isopropylated TPPs that were identified in the liver are indeed capable of inhibiting mouse CAR.

In contrast to mouse CAR, human CAR was activated 2.5–5.5-fold by all compounds except with *p*-iPrX3 which inhibited the activity by 45%. There was a structure-dependent trend in human CAR activity in that *ortho*-substitution gave 30% higher activities that the *meta*- or *para*-substitution, and increasing the number of substituents resulted in a progressive decline in activity. Mouse PXR was weakly activated by triaryl phosphates (≤1.5-fold). However, one or two substituents and *ortho*-substitution yielded more active compounds than other isomers were ($P < 0.05$), in analogy with human CAR. Human PXR was activated by 2–4-fold by all triaryl phosphates. Now, there was no clear structure-dependent trend in the activity, with *m*-iPrX3 yielding the highest response. We also tested mouse and human PPARα receptors, but did not see clear signs of receptor activation or suppression (data not shown). Dose–response studies with TPP and *o*-iPrX3 indicated that the half-maximal concentration for mouse CAR inhibition by *o*-iPrX3 was less than 1 μM (Fig. 4A). Human CAR and human PXR were activated half-maximally at concentration slightly below 1 μM (TPP) or about 2 μM (*o*-iPrX3) (Fig. 4A and B). For mouse CAR and mouse PXR activation, these concentrations could not be accurately estimated due to their modest responses. In summary, various triaryl phosphates are rather potent activators of human CAR and PXR at 10 μM concentration while the responses of mouse PXR are weaker and those of mouse CAR vary from strong inhibition to activation. These species-specific responses further confirmed the established differences between human and rodent CAR and PXR receptors [9,17]. Structure-dependent trends in activity were evident for all receptors except for human PXR. This is not surprising in the view of the flexible binding site of human PXR [17].

Figure 5 shows the response of human steroid hormone receptors GR, PR, AR, and ERα to triaryl phosphates. Because the steroid hormone receptors are highly conserved (>90% similarity) in their ligand-binding domains and thus show practically identical ligand specificities across species, we did not consider the unavailability of mouse receptors a major drawback. *Ortho*- and *para*-substituted phenyl phosphates were chosen because of their distinct spatial conformations and the greatest differences in activities in Fig. 3, along with the parent compounds TTP and TPP. In the absence of any added agonist (Fig. 5A), human GR was inhibited 20% by TPP only. Human PR and ERα did not show any clear agonism or antagonism by triaryl phosphates. Human AR activity was decreased (40–50%) by TPP and *p*-iPrX1 ($P < 0.05$), and there was a tendency of suppression with *ortho*-substituted compounds *o*-iPrX1 and *o*-iPrX2 as well ($P < 0.1$). When

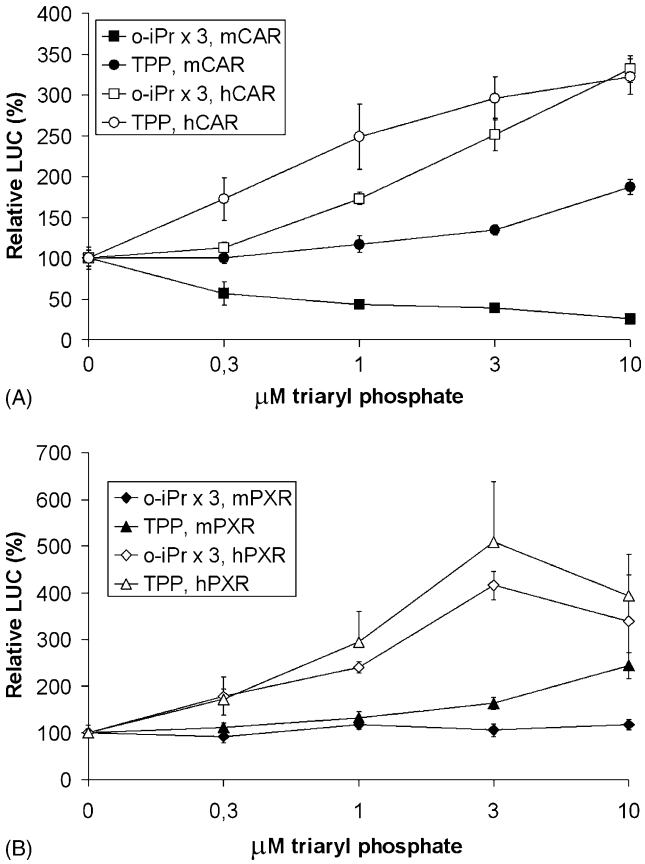


Fig. 4. The dose–responses of mouse and human NRs CAR and PXR to triaryl phosphates. HEK293 cells were first transfected with mouse (filled symbols) or human (empty symbols) NRs CAR (A) or PXR (B) and their reporter genes. Transfected cells were then treated with indicated concentrations (0–10 μM) of TPP (circles in A, diamonds in B) and *o*-iPrX3 (squares in A, triangles in B) and analyzed as in Fig. 3.

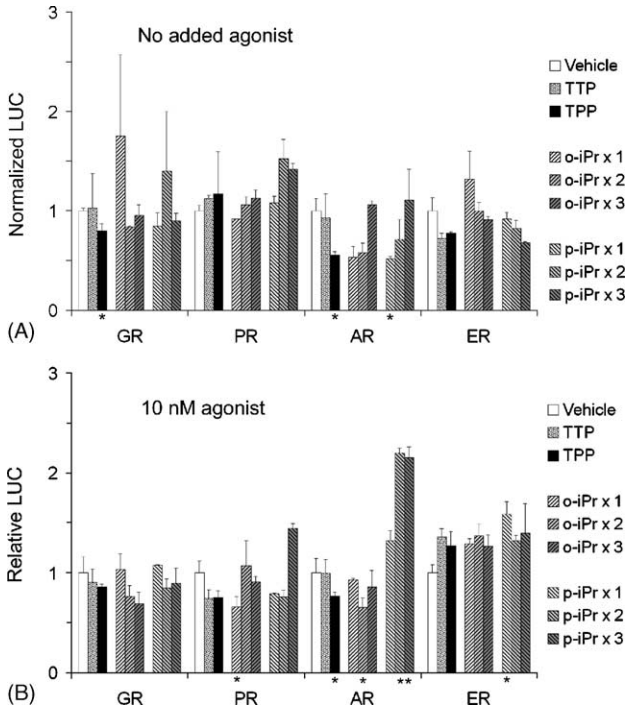


Fig. 5. The effects of triaryl phosphates on human GR, PR, AR, and ERα. COS-1 cells were transfected with indicated NRs *plus* their reporter genes and treated with vehicle or triaryl phosphates (10 μM) in the absence (A) or presence (B) of respective steroid agonist (10 nM). The data shown are means ± SE from three independent experiments, and expressed as relative to vehicle (set to unity). The columns are grouped in sets of three: vehicle (empty), TTP (stippled) and TPP (filled); three *ortho*-substituted (hatched-up) and three *para*-substituted (hatched-down) triaryl phosphates. In the last two groups, the background color reflects the number of alkyl substituents (N = 1, empty; N = 2, light gray; N = 3, dark gray). The asterisks (*) below the columns indicate statistically significant difference in comparison with vehicle ($P < 0.05$). The activation of ΓP, ΠP, AP, αvδ EPα by their cognate ligands dexamethasone, progesterone, testosterone, and estradiol (at 10 nM) were 24.9 ± 4.0-fold, 9.6 ± 1.2-fold, 29.8 ± 4.1-fold, and 3.3 ± 0.26-fold, respectively.

these receptors were assayed in the simultaneous presence of agonists (Fig. 5B), no change in dexamethasone-induced GR-dependent activities took place. Progesterone-induced PR-dependent activity was decreased only by *o*-iPrX1 (30%). Testosterone-induced AR-dependent activity was lowered by 30–40% by TPP and *o*-iPrX2 ($P < 0.05$), and di- and tri-*para*-substituted phenyl phosphates increased this activity by over 100%. Only *p*-iPrX1 increased the estradiol-induced ERα activity by 32%. In addition to these results, vitamin D receptor was also tested but no discernible effects by triaryl phosphates took place (data not shown). In summary, GR, PR and ERα were rather insensitive to triaryl phosphates regardless of the presence of receptor agonists. On the other hand, AR was consistently inhibited by TPP and *ortho*-substituted di-isopropylated phenyl phosphate.

4. Discussion

To understand better the physiology of the mouse CAR, we set up a purification scheme to identify for its putative endogenous inhibitor from liver tissue. Surprisingly, we identified isopropylated TPPs in the active fractions. Synthesis of the identified compounds and their congeners proved that these triaryl phosphates could indeed inhibit mouse CAR. Because the identified compounds could not be accounted for by any endogenous biochemical pathways and they are widely produced, we concluded that their presence in the liver was caused by an environmental release. However, we could not clearly identify a source in the diet or in the housing or care of the animals, and could therefore not rule out a one-time contamination, e.g. somewhere along the chow processing. Because our screen for the endogenous CAR inhibitor was based on a functional assay without any prior bias to a chemical class, the presence of isopropylated TPPs has probably masked any effect by any endogenous inhibitor. It is also possible that the endogenous inhibitor is unstable or present in sufficiently low amounts to avoid detection, especially because the extraction procedure was rather complicated and unoptimized for any particular class of chemicals.

Further studies are thus warranted to identify the true inhibitor for mouse CAR.

Triaryl phosphates are a group of organophosphates that are widely used as flame retardants, plasticizers, and in hydraulic fluids [33]. Their estimated annual production is in the order of 50,000 tons [34,35]. TPP and isopropylated phenyl phosphate ranked among top three flame retardants in use in Sweden during the year 1999 [36], and both are used in high concentrations in electronic equipment [36,37]. As a result of leakage and leaching, triaryl phosphates have been found to contaminate soil and water [36,39] and they are present even in air at low concentrations [38]. Because of their lipophilicity ($\log P_{ow} \approx 5$), they can be absorbed and bioaccumulated about 1000-fold [34,35,40,41]. Their biological half-lives are in the range of 20–80 hr in rats and fish, hydrolysis being the main degradative route [40,42]. Preliminary incubations of isopropylated TPPs in liver homogenates indicated that they are quite resistant to degradation.[1]

Apart from the established neurotoxicity of tri-*ortho*-cresyl phosphate and other *ortho*-substituted triaryl phosphates [43], the knowledge about the toxicity of other triaryl phosphates is rather limited [34,35,44]. Tricresyl phosphate has been prioritized to human reproductive field studies [45] based on the size of potentially exposed population, volume of chemical production, and reproductive defects detected in the rodent bioassays. These reproductive abnormalities included decreased fertility, sperm count, and sperm motility [46,47], hypertrophy of adrenocortical and ovarian cells, decreased testicular weights, and degeneration of seminiferous tubules [46,48,49]. Inhibition of cholesterol ester breakdown and associated increase in serum cholesterol and a decrease in estradiol levels were also seen [50,51]. All these data suggest changes in steroid hormone balance and/or action of the exposed animals, but the contributing mechanisms have remained unclear.

Our data show that xenobiotic-responsive NRs can be affected by triaryl phosphates, with EC_{50} values of the responses around 1 μM and defined structure–activity relationships for CAR and PXR. Human CAR and PXR were consistently activated by triaryl phosphates while mouse CAR and PXR responded variably. This finding implies that especially the human target genes *CYP2B6* and *CYP3A4* for CAR and PXR might be activated. Because these genes code for efficient steroid hydroxylases [9], one could expect a change in the pattern of steroid metabolism, especially after a high-dose or extended exposure. In this respect, an increase in hepatic CYP-mediated activities has been reported in rats injected with a commercial triaryl phosphate mixture [52]. It is noteworthy that CAR and PXR regulate many other genes in addition to CYPs and steroid conjugating enzymes [53], thus increasing the number of biological targets potentially affected by triaryl phosphates. The differential response of human and mouse receptors would suggest difficulties in extrapolation of animal toxicity studies to human situation. Long-term therapy for epilepsy and tuberculosis has exposed humans to CAR and/or PXR activators such as carbamazepine, phenytoin, and rifampicin, and this information would aid in understanding potential effects of triaryl phosphate activation of CAR and PXR. However, there is very little information, e.g. on the blood levels of sex steroids or any reproductive abnormalities in these patients that could be directly linked to drug exposure. There seems to be an increase in metabolism of estradiol in females and no change or decreases in testosterone levels in males (e.g. [54–56]). These findings lend some support to the notion that PXR and/or CAR activation may change endogenous hormone levels, but it is not known whether exposure to triaryl phosphates, e.g. in the workplace, is severe enough for such changes. The estimates of average daily intake of some triaryl phosphates [34,35] for a 70 kg person are in the order of 1000 ng per day, suggesting that the general population is at low or negligible risk for such effects. However, bioaccumulation factors of about 1000 and low rates of degradation for substituted triaryl phosphates [34] suggest that (sub)micromolar values may be possible in areas with high contamination or high dose exposure. Even if the general population is not exposed to (sub)micromolar concentrations, such doses are frequently found in animal toxicity studies. So, our results may find application in interpreting those studies.

A more direct connection to abnormalities in male reproduction may stem from the effects of triaryl phosphates on the AR. Both TPP and *o*-iPrX2 inhibited AR activity in the presence of testosterone. Some suppression of the unliganded AR was also seen, perhaps due to some basal activity of the AR remaining in the absence of testosterone. The compounds *p*-iPrX1 and *p*-iPrX3 increased the AR activity only in the presence of testosterone, but the mechanism of such co-activation remains unclear at the moment. Even though these effects on AR took place at rather high triaryl phosphate concentration (10 μM), they were specific in the sense that other steroid hormone receptors were refractory to these compounds. No change in AR activation was noted with TTP, which represents, however, only the tri-*para*-substituted form of 11 possible isomers of tricresyl phosphate [34], the suspected reproductive toxicant. More definitive studies in the response of AR are therefore clearly needed. Similarly, many commercial triaryl phosphates that are in use are proprietary mixtures of various congeners and their isomers. This renders prediction of their effects (especially on mouse CAR) highly complicated.

We were not equipped, at this time, to conduct any functional tests on whether triaryl phosphates can indeed affect steroid metabolism and/or AR signaling in human hepatocytes or other cells. Nevertheless, our results help to create new hypotheses and mechanistic explanations for the observed adverse biological effects of triaryl phosphates. In conclusion, our results confirm the wide-spread

[1] Saarela M, Auriola S, Honkakoski P, unpublished experiments.

contamination by triaryl phosphates, and indicate for the first time that triaryl phosphates are efficient activators of human CAR and PXR, the main regulators of steroid hormone-metabolizing CYP and conjugating enzymes, and suggest biological pathways that might be affected by triaryl phosphates.

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